University of Alberta

Physical genome map of *Haemophilus ducreyi* KC1 (CIP 542^T)

by

Hazel Talbot

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Medical Microbiology and Immunology

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DEDICATION

This thesis is dedicated to my husband Randy and to my daughters Alexandra and "Sarah".

ABSTRACT

Haemophilus ducreyi is the causative agent of chancroid, an ulcerative sexually transmitted disease. Little basic information regarding the genetics of *H. ducreyi* is available. Therefore, a physical restriction map of the genome of the type strain of the species, CIP 542, was constructed on which newly discovered genes can be placed and which can be used for epidemiological and taxonomic purposes. KC1, a commonly used strain of CIP 542 was chosen for physical mapping. Mapping utilized pulsed-field gel electrophoresis techniques and the following strategies: (I) the use of four restriction endonucleases *Asc*I, *Not*I, *Sfi*I and I-*Ceu*I which cut the genome at 12, 9, 4 and 6 sites, respectively (II) double digests (III) sequential reciprocal digests (IV) cross-hybridization. The chromosome was found to be circular 1771 ± 43 kb with six copies of 16S and 23S rRNA. Twenty-six other genes were positioned on the map, including those associated with virulence; *hhdB,A* (hemolysin gene), *hupA* (hemoglobin-binding outer membrane protein gene), *momp* (OMP A homologue gene) and *ftpA* (fine-tangled-pilus gene).

The pulsed-field electrophoretic profile of KC1 was also found to be significantly different from the ATCC strain of CIP 542. Thus, at least 2 different strains have been designated CIP 542. This may help resolve some controversies in the literature regarding virulence of this strain.

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LIST OF ABREVIATIONS

BSA	bovine serum albumin
C	cytosine
CDC	Centers for Disease Control and Prevention
CHEF	contour-clamped homogeneous electric field
CIP	Centre Institute Pasteur
CPE	cytopathic effect
DNA	deoxyribonucleic acid
dnaK,J	heat shock protein genes
dCTP	dideoxy-cytosine-triphosphate
EDTA	disodium ethylene diamine tetraacetate
FIGE	field inversion gel electrophoresis
ftpA	24K pilin monomer, (fine tangled pilus gene)
G	guanine
groES/EL	heat-shock protein genes
h	hours
HaCaT	keratinocyte cell line
HFF	human foreskin fibroblasts
hhdB,A	hemolysin gene
hlp	28K outer membrane lipoprotein gene
hlyX	homolog of fumarate nitrate repressor gene
hupA	hemoglobin binding protein gene
kDa	kilodaltons
LOS	lipooligosaccharide

mg	milligram
min	minutes
ml	millilitre
mM	millimolar
MOMP	major outer membrane protein
momp	major outer membrane protein (OmpA homologue) gene
mutS	DNA mismatch repair protein gene
opaA	cell envelope protein gene associated with colony opacity in *H. influenzae*
OMP	outer membrane protein
pal	18K OMP (PAL homologue) gene
pbpA	penicillin binding protein gene
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
16S rRNA	16S ribosomal ribonucleic acid
23S rRNA	23S ribosomal ribonucleic acid
s	seconds
TAFE	transverse alternating field electrophoresis
TBE	tris borate EDTA buffer
TE	tris EDTA buffer
µg	microgram
µl	microlitres

1. INTRODUCTION

Haemophilus ducreyi is a fastidious gram-negative bacillus that causes the sexually transmitted genital ulcerative disease known as chancroid or soft chancre. The genital ulcerative diseases are common worldwide and include syphilis (hard chancre), genital herpes, chancroid, lymphogranuloma venereum and donovanosis. The causes of genital ulcerative diseases are strikingly different worldwide with genital herpes being the most common in the USA and Europe and chancroid being the most common in tropical countries (Morse S.A., 1989). There was a paucity of information on *Haemophilus ducreyi* in the literature before the 1980's. However, over the past two decades the number of reported cases has increased significantly in the United States and Europe and urban epidemics have been reported in North America (Albritton W.L.,1989). Additionally, genital ulcerative diseases are co-factors in the transmission and acquisition of HIV (Tyndall *et al.*, 1996, Pepin *et al.*, 1992). These two factors have led to increasing interest in *Haemophilus ducreyi*.

The goal of this project was to create a physical restriction map of the type strain of *H. ducreyi*, CIP 542^T. This strain has been defined as avirulent due to its inability to produce lesions when inoculated intradermally in a rabbit (Feiner et al., 1945). The map would have varied applications as follows:

1) It would provide basic information on the genetic organization of the type strain of the species. It would establish genome size and the circularity of the chromosome.

2) Newly discovered genes could be mapped. Knowing the position of the gene on the chromosome helps to confirm that the gene is novel.

Additionally, its position with respect to its neighbours gives clues as to its function and/or regulation. Finally, knowledge of map position allows its manipulation in genetic experiments, for example, in isolating deletions and in localized mutagenesis.

3) Physical genome maps are important tools for comparing strains. They provide more information than restriction enzyme fragment profiles in that the arrangement of the fragments around the chromosome can be compared. Knowing the gene order on the chromosome and the locations of genes with respect to restriction sites allows another level of comparison of strains. Comparisons of maps of virulent and avirulent strains is a means for studying mechanisms of virulence. In the field of epidemiology, maps of strains from various geographical regions can be compared to study outbreaks of the disease. Taxonomy is an area that needs to be studied for this organism and the comparison of physical genome maps and gene order would be helpful in placing strains of *H. ducreyi* within a new genera. Finally chromosomal rearrangements among strains of *H. ducreyi* could be discovered using genome maps and so begin research into genetic diversity within the species.

2. *HAEMOPHILUS DUCREYI*

2.1. Taxonomy

The taxonomy of *H. ducreyi* is in question. *H. ducreyi* belongs to the Family *Pasteurellaceae*, which contains the genera *Pasteurella*, *Haemophilus* and *Actinobacillus*. This is a diverse group and studies using DNA-DNA hybridization (Mutters, *et al.*, 1985), DNA-RNA hybridization (De Ley *et al.*, 1990) genetic transformation (Albritton *et al.*, 1989), DNA hybridization and S1 Nuclease treatment (Casin *et al* ., 1985) have shown that the 3 genera do not adequately accommodate strains assigned to this family. The study by Casein *et al* showed that 25 strains of *H. ducreyi* from various geographic regions were 80-100% related to one another but were only 0-6% related to 17 strains of established *Haemophilus* species. The most recent study to further clarify the taxonomic organization of the *Pasteurellaceae* used data from 16S rRNA sequencing to create 2 new clusters, in addition to the three clusters containing the genera *Pasteurella*, *Haemophilus* and *Actinobacillus* (Dewhirst *et al.*, 1992). Three strains of *H. ducreyi* (including the type strain CIP 542) were included in the study involving 54 representative strains within the family *Pasteurellaceae*. *H. ducreyi* was placed in cluster 4, separate from the true haemophili which were placed in cluster 1.

Originally *H. ducreyi* was placed in the genus *Haemophilus* due to a requirement for hemin. By definition, the species *Haemophilus* includes only those organisms which are gram-negative rods or coccobacilli that require X (hemin) or V (nicotinamide adenine dinucleotide) factor (Killian *et al.*, 1984). In addition it has a guanine-plus-cytosine (G+C) content of 38 mol%, similar to that of *Haemophilus* species (Albritton, 1989), the haemophili

having a G+C content of 37 to 44 mol%. Codon usage was also found to be similar to *H. influenzae* in that *H. ducreyi* used U-or A-rich codons 79% of the time (Parsons *et al.*, 1992). Taking into account the data from DNA hybridization studies and 16S rRNA sequencing, renaming of *H. ducreyi* would seem appropriate along with a reorganization of the family *Pasteurellaceae*.

2.2. Clinical features

A break in the epithelium is thought to be the portal of entry and scarification of the skin is required to produce experimental infection of the forearm (Spinola *et al.*, 1994). The usual incubation period is between 4 and 7 days. The lesion begins as a small inflammatory papule surrounded by a narrow region of erythema. In the next 2 to 3 days a pustule forms, ruptures and leaves a sharply circumscribed ulcer with ragged undermined edges lacking induration (Morse, 1989). The base of the ulcer has a granular appearance (unlike the syphylitic ulcer which has a smooth base) and may be filled with a necrotic purulent exudate. The ulcers are very vascular and the granulomatous base is friable, bleeding easily on scraping. In males the lesion is always painful. In females the lesion may be less painful or painless if located internally (e.g cervix). In up to 50% of cases, painful, tender inguinal lymphadenopathy develops which may lead to a chancroidal bubo which ruptures to form an ulcer. Systemic infections are not associated with this disease.

The histological features of the ulcers include 3 zones which have been well documented (Sheldon *et al.*, 1946). The superficial zone represents the base of the ulcer having degenerated polymorphonuclear granulocytes, red cells,

fibrin and microorganisms. The middle zone is broader with edematous inflamed tissue with numerous, dilated vessels arranged vertically. A marked endothelial overgrowth occurs. The deep zone consists of dense infiltrate of plasma cells and fewer lymphocytes.

In the absence of antimicrobial therapy, ulcers persist for weeks to months. The CDC recommends chancroid be treated with one of erythromycin, azithromycin or ceftriaxone (Trees *et al.*, 1995).

2.3. Laboratory diagnosis

H. ducreyi is a small, gram-negative, nonmotile rod, 0.5 to 0.6 μm in width by 1.6 to 2.0 μm in length. Gram-stained smears from lesions reveal clumps of cells and short streptobacillary chains or "railroad tracks". Cultivation is on enriched medium (e.g. chocolate agar) with supplements such as IsoVitalex (BBL). X factor (hemin) is required for growth. Inoculated plates are incubated at 33°C in a humid atmosphere with 5% CO_2. Colonies appear in 24 to 72 h and can be easily pushed across the agar with a loop. Gram stain may show a typical "school-of-fish" arrrangement. Carbohydrate fermentation tests are not useful in identifying *H. ducreyi,* only in differentiating it from other *Haemophilus* species. It is negative for the porphyrin and catalase test but positive for alkaline phosphatase and nitrate reductase. It has a positive oxidase test using tetramethyl-p-phenylenediamine (Morse, 1990).

Nonculture diagnostic tests show promise in improving the diagnosis of chancroid. Recently monoclonal antibodies have been described that are species specific (Alfa *et al.*, 1992, Ahmed *et al.*, 1995). Polymerase chain reaction (PCR) assays have also shown great potential for improving the

diagnosis of chancroid (Chui *et al.*, 1993, Johnson *et al.*,1994, Parsons *et al.*, 1995, Orle *et al.*, 1996).

2.4. Epidemiology

H. ducreyi was a significant problem in military personnel during both world wars but was rarely seen in the 1960's and early 1970's in North America. Chancroid is prevalent in Africa, Asia and Latin America and is considered a major public health problem. In some areas of the United States it is a common cause of genital ulcers (Orle *et al.*, 1996). In 1947 the reported number of cases peaked in the United States at 9,515 cases and then declined through the mid 1980's. Another peak occurred in 1987 when 4,986 cases were reported followed by another decline to 1,399 cases in 1993 (Centers for Disease Control and Prevention, 1994). In Canada, chancroid outbreaks are sporadic with an epidemic occurring between 1975 and 1977 in Winnipeg (Hammond *et al.*, 1980). Outbreaks have been generally associated with prostitution and a lack of circumcision in men. The disease is generally found within the heterosexual population. (Jones *et al.*, 1990, Blackmore *et al.*, 1985). Thus, while chancroid had been considered to be uncommon in North America, it is reemerging as a significant STD which is now endemic in the United States.

2.5. Strain typing

The epidemiology of chancroid has not been well defined since typing systems are not well developed. Several typing schemes have been proposed. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of sarcosinate-insoluble membrane preparations has been used to compare the outer membrane protein profiles (Odumera *et al.*,1983). Seven patterns

were observed using this method. The same method used to characterize strains isolated in Southeast Asia showed 98% of the strains belonged to three groups based on outer membrane patterns, suggesting this method may not adequately differentiate strains (Taylor *et al.*, 1985). An indirect immunofluorescence assay (Slootmans *et al.*, 1985) developed by immunizing rabbits with untreated whole cells of *H. ducreyi* suggested that unique antigenic determinants were present and nine types were identified. A typing system using enzyme profiles was also described with three biovars being identified based on L-ornithine and L-alanyl-L-phenylalanyl-L-proline arylamidase (Van Dyck *et al.*, 1987).

Ribotyping schemes have been used successfully. Using *Hind*III and *Hinc*II banding patterns and probing 132 strains from various geographical areas with 16S and 23S RNA from *E. coli* yielded 12 ribotypes with *Hind*III and 5 ribotypes with *Hinc*II (Sarafian *et al.*, 1991). Another study identified 10 *Hind*II, 3 *Hinc*II, 7 *Bst*EII and 10 *Bgl*I ribotypes among 30 strains from diverse geographical areas (Brown *et al.*,1993). Pillay *et al* identified 13 ribotypes using 30 strains with *Bgl*I, *Hind*III, *Hind*II, and *Bst*EIIA and concluded that a diversity of ribotypes existed (Pillay *et al.*, 1996). A combination of ribotyping and plasmid analysis has also been used successfully to type strains. Plasmid analysis alone is not useful since different strains may have identical plasmid profiles and plasmids may be also be transferred by conjugation (Flood *et al.*, 1993), while some strains have no plasmids.

Immunotyping has been used to show antigenic diversity. Antisera were pooled from 238 patients and cross reacting antibodies removed by absorption with antigens from closely related bacteria. Two specific

antigenic determinants, an immunodominant 28 kDa and a 30 kDa protein, were identified. Using both proteins as immunotypic markers, 7 different types were identified in a total of 63 isolates (Roggen *et al.*, 1992).

A typing system needs to be "sensitive enough to distinguish organisms that are similar but not identical" and "be of proven value based on epidemiologic investigation" (Pfaller, 1991). More research needs to be done in these areas before any typing scheme can be determined to be useful for *H. ducreyi*.

2.6. Human immune response

Studies of the host (human and rabbit) response to infection with *H. ducreyi* have demonstrated IgM, IgG, and IgA antibodies to multiple antigens. The antibodies are directed at the outer membrane protein and lipooligosaccharide (Desjardins *et al.*,1992, Schalla *et al.*,1986, Roggen *et al.*, 1992, Roggen *et al.*, 1994, Alfa *et al.*, 1993). This humoral immune response is not protective as is evidenced by repeat infections and serial autoinoculations. Failure of HIV-infected patients to respond to standard therapy for chancroid (Tyndall *et al.*, 1993) and demonstration of activated T-cells and macrophages in the ulcers of experimental human volunteers (Spinola *et al.*,1994) suggests an important role for cell-mediated immunity in chancroid.

2.7. Models for study of pathogenesis

There have been numerous *in vitro* studies using various cell lines to compare *H ducreyi* adherence, invasion and cytopathic effect (CPE), some

involving comparison of virulent and avirulent strains (Alfa, 1992, Alfa *et al.*, 1993, Lammel *et al.*, 1993). A recent review of the literature summarizing *in vitro* studies found that adherence occurred in all 13 cell lines used, invasion of the cell line in 6 out of 12 studies and 9 out of 16 reported CPE (Trees *et al.*, 1995). In a study involving binding of *H. ducreyi* to human keratinocytes, the first host cells encountered in infection, 15 to 23% of the inoculum bound compared to 1% for *E. coli* or *Moraxella catarrhalis* (Brentjens *et al.*, 1994).

A variety of animal models exist with some showing promise in the study of *H. ducreyi* pathogenesis. Intradermal injection into rabbits has been the classical test of *H. ducreyi* virulence (Hammond *et al.*, 1978) but a large inoculum is required, replication of the organism does not appear to be necessary for disease development and the resulting lesions do not resemble those of human chancroid ulcers (Tuffrey *et al.*, 1988, Purcell *et al.*, 1991). Moreover, "avirulent" strains in this model retained the ability to produce lesions in humans (Trees *et al.*, 1992). An intradermal mouse model which mimicked human ulcers was reported (Tuffrey *et al.*, 1988) but was subsequently shown to be non-specific (Tuffrey *et al.*,1990). Growth in mouse subcutaneous chambers has been suggested as a means of studying virulence determinants expressed *in vivo* (Trees *et al.*, 1991). Introduction of a temperature-dependent rabbit model is a major advance in pursuing the molecular pathogenesis of *H. ducreyi.* A productive infection requiring viable replicating organisms can be established in rabbits with an intradermal inoculum three times less than in the standard rabbit model by reducing the ambient temperature to 15-17^{o}C (Purcell *et al.*, 1991). Induction of significant protective immunity has been demonstrated in this model using whole cell envelopes (Hansen *et al.*, 1994) or purified pilin (Desjardins *et al.*,

1995) and the results suggest that it may be possible to develop an effective chancroid vaccine. However, no protection has been demonstrated upon rechallenge in a human volunteer model (Spinola *et al.*, 1994) This may be related to the short duration of experimental human infection or could indicate a fundamental difference in the response to *H. ducreyi* in humans compared with rabbits. Primate and swine models have also been described. Adult pigtailed macaques were experimentally infected and produced a disease closely resembling chancroid, developing lesions in 6 to 12 days, humoral antibodies in 1 week and inguinal lymphadenopathy in some cases (Totten *et al.*,1994). Domestic swine were also infected with *H. ducreyi* producing ulcers histologically resembling human lesions (Hobbs *et al.*, 1995). In both cases, *H. ducreyi* was recovered from lesions in 17 to 20 days after inoculation.

2.8. Virulence factors

Most research on the pathogenesis of *H. ducreyi* has centered on lipooligosaccharide (LOS), cytotoxin, outer membrane proteins (OMP) and the role of pilus-like structures. Recently, genes have been described which advance our knowledge of virulence mechanisms.

2.8.1. fine tangled pilus

Pili are present on most gram-negative bacteria and are adhesins. The fine tangled pilus of *H. ducreyi* is composed of a unique major subunit and lacks a leader peptide (Brentjens *et al.*, 1996). *H. ducreyi* pili bind to laminin (Abeck *et al.*,1992, Westerlund *et al.*, 1989) although their role in pathogenesis still needs to be determined. The gene encoding *H. ducreyi* pilus (*ftpA*) has been cloned (Brentjens *et al.*, 1996).

2.8.2. hemoglobin binding protein

The ability of *H. ducreyi* to cause disease is related to its ability to acquire nutrients. The bacteria require large amounts of heme or hemoglobin *in vitro* suggesting that protoporphyrin-iron complexes are necessary to maintain virulence. The *hgbA* gene (Elkins *et al.*, 1995) or *hupA* gene (Stevens *et al.*, 1996) encodes a 108 kDa outer membrane protein that is essential for the organism to utilize exogenously supplied heme and for the expression of full virulence in an animal model.

2.8.3. hemolysin

Hemolysins are able to lyse many cell types or interfere with their cellular function (Welch, 1991) and therefore could be important virulence factors. The presence of a hemolysin suggests that *H. ducreyi* is able to release hemoglobin from erythrocytes to aquire heme (Stevens *et al*, 1996). The hemolysin could also act upon epithelial and inflammatory cells (Totten *et al.*, 1995). It has been shown that *H. ducreyi* hemolysin is responsible for the cytopathic effect on human foreskin fibroblasts (HFF) cells and keratinocyte (HaCaT) cells (Palmer *et al.*, 1996). The hemolysin gene cluster has been described (Palmer *et al.*, 1995) which contains two genes. The *hhdB* gene encodes an outer membrane protein required for the activation and secretion of hemolysins and have homology to the *ShlB* and *HpmB* protein genes produced by *Serratia marcescens* and *Proteus mirabilis*. The *hhdA* gene encodes a pore-forming toxin similar to the ShlA and HpmA hemolysins produced by *S. marcescens* and *P. mirabilis*.

2.8.4. major outer membrane protein (MOMP) (OMP A homologue)

The MOMPs are usually classical porins in gram-negative bacteria having important immunogenic determinants and are usually the most abundant protein in the outer membrane (Hancock, 1986). The *H. ducreyi* MOMP, with a molecular weight of 39 to 42 kDa, does not have the characteristics of the classical porins, and is related to the OmpA proteins of members of the families *Enterobacteriaceae*, *Neisseriaceae* and *Pasteurellaceae* (Spinola *et al.*, 1993). Although its role in virulence is speculative it may play a role in serum resistance (Weiser *et al.*, 1991) or it may bind antibodies that block serum bactericidal activity as has been described for gonococcal protein III (Rice *et al.*, 1986). Recently, the gene encoding the *H. ducreyi* MOMP has been cloned (Hiltke *et al.*, 1995).

2.8.5. heat-shock proteins

The *groE* operon encodes heat shock proteins which are major antigens and evoke humoral and cellular responses in infected hosts (Young *et al.*, 1990). The heat shock response involves the coordinate expression of a group of highly conserved proteins. The GroES and GroEL proteins of *E. coli* are molecular chaperones during protein folding and translocation (Gross *et al.*, 1990). The *H. ducreyi groES/EL* genes have been cloned and sequenced and it has been postulated that these genes play a role in the tissue destruction found in chancroid (Parsons, *et al.*, 1992).

2.8.6. lipooligosaccharide

The lipopolysaccharide of *H. ducreyi* is rough, lacking the repeating O-antigens present in enteric organisms. It is similar in size and structure to other mucosal pathogens and so is referred to as lipooligosaccharide.

Intradermal injection of *H. ducreyi* LOS produces skin abscesses in rabbits suggesting that it may play a role in pathogenesis (Campagnari *et al.*, 1991). It may also play a role in the evasion of the host immune response since the LOS has a terminal N-acetyllactosamine that undergoes sialylation and so mimics the human paragloboside, a precursor to a major human blood group antigen (Melaugh *et al.*, 1994). It is also a virulence factor of *N. gonorrhoeae* which converts the organism from serum sensitive to serum resistant (Mandrell *et al.*, 1988). Recently, the gene encoding *H. ducreyi* CMP-NeuAc synthetase, an enzyme involved in sialylation, was cloned and sequenced (Tullius *et al.*, 1996).

2.9. Genetics

Little is known about genomic organization and the genetics of *H. ducreyi* although antibiotic resistance plasmids are widespread (Morse,1989, McNicol *et al.*, 1984). *H. ducreyi* both donates and receives conjugative plasmids from members of the *Pasteurellaceae* and *Enterobacteriaceae* which appear to share a common gene pool (Albritton, 1989, Brunton *et al.*, 1986, Albritton *et al.*, 1982). Expression of *H. ducreyi* genes in *E. coli* (Parsons *et al.*, 1992, Hansen *et al.*, 1992) and a shuttle plasmid stable in both species (Wilson *et al.*, 1989, Dixon *et al.*, 1994) have been described. Transposon mutagenesis using Tn*10*, Tn*916* and Tn*1545*-D3, allelic exchange and transformation by electroporation have been reported (Hansen *et al.*, 1992, Stevens *et al.*, 1995).

Recently a rudimentary physical and genetic map of the virulent *H. ducreyi* strain 35000 was constructed using the enzymes I-*Ceu*I and *Sfi*I (Hobbs *et*

al, 1996). The genome size of strain 35000 is 1757 kb and it contains six ribosomal RNA operons.

3. GENOME MAPS

3.1. Strategies for creating genome maps

There are three strategies for creating genome maps; genetics, ordered libraries and PFGE. Genetic maps provide distances measured in units derived from recombination frequencies. The traditional genetic method was used to create maps of *Escherichia coli* (Bachman, 1983), *Salmonella typhimurium* (Sanderson *et al.*, 1988), *Pseudomonas aeruginosa* (Holloway *et al.*, 1990) and *Bacillus subtilis* (Henner *et al.*, 1980). The genetic method necessitates the use of genetic transfer systems and a large number of genetic markers. Therefore, the study of many important bacteria is not possible by the genetic method. Ordered libraries are cloned DNA fragments ordered in the sequence of the chromosome. (Fonstein *et al.*, 1995). Kohara obtained the first physical map of *E. coli* by this method (Kohara *et al.*, 1987). PFGE enables the separation of DNA up to several megabases in size so that restriction sites can be mapped relative to each other without the use of genetic methods. *E. coli* was the first bacterium to be mapped using this method (Smith *et al.*, 1987).

3.2. PFGE and physical genome analysis

Pulsed-field gel electrophoresis and its use in mapping was first described in an article by Schwartz and Cantor in 1984 (Schwartz *et al.*, 1984). PFGE is a powerful technique that has been applied to many organisms (Cole *et al.*, 1994). The principle of gel electrophoresis is the same for both conventional electrophoresis and PFGE. DNA samples are placed in a solid matrix, agarose, and an electric field is applied inducing the molecules to move through the gel. In the absence of an electric field, DNA molecules exist in a relaxed coiled state. Elongation occurs upon application of the electric field

as the molecule aligns with the electric field and migrates toward the anode. Size separation is achieved with conventional electrophoresis since small molecules move more easily through the gel, however, only molecules smaller than 20 kb can be separated. In contrast, the limit of pulsed-field gel separation is approximately 10,000 kb (Birren *et al.*, 1993).

The principle of PFGE is based on the fact that after the removal of the electric field, the DNA returns to its relaxed coiled state. The rate of relaxation is dependent on the length of the DNA. By periodically changing the orientation of the electric field, the molecule goes through a relaxation phase, followed by a reorientation in the direction of the new field. The time it takes for this to occur is size dependent. The term "pulsed-field" is used when more than one electric field is applied and the fields are activated alternately. The term switch interval or pulse time refers to the amount of time each of the alternating electric fields is active. The nomenclature used to describe the various PFGE systems (e.g. field inversion gel electrophoresis (FIGE), transverse alternating field electrophoresis (TAFE)) refers to a particular aspect of the hardware (e.g. electrode geometry) (Birren *et al.*, 1993). The equipment used for the physical map of *H. ducreyi* CIP 542 was a clamped homogeneous electric field electrophoresis (CHEF) system. Straight lanes can only be achieved using a homogeneous field. Twenty-four electrodes are "clamped" in a hexagonal array generating a 120^{0} reorientation angle (Chu *et al.*, 1986). The major advantage of using the CHEF system is its ability to separate large numbers of DNA samples in straight lines.

The method used by Schwartz and Cantor for preparing large molecules of DNA in agarose was as revolutionary as the PFGE itself. Care must be taken

when preparing DNA larger than 500 kb to protect the molecules from mechanical breakage from shear forces and nucleolytic degradation. Cells are imbedded in agarose to protect the DNA yet allowing the flow of solutions and non-DNA components through the agarose. Cells are solubilized and digested in this manner leaving intact chromosomal DNA embedded in the agarose. The process is done in the presence of detergent and EDTA to prevent degradation of DNA by nucleases (Schwartz *et al.*, 1984).

Physical genome analysis for the purpose of producing a low resolution map requires restriction enzymes which will digest the genome in a limited number of sites, ideally 1 to 20 (Fonstein *et al.*, 1995) and a combination of enzymes that produce a distance of 100-200 kb between restriction sites (Cole *et al.*, 1994). A guideline in the initial selection of enzymes is that the frequency of cutting depends on the length of the recognition site and on base composition, therefore enzymes with GC-rich restriction sites may be suitable for AT-rich genomes since they will digest infrequently (Birren *et al.*, 1993). Also, restriction enzymes that are involved in intron processing have large restriction sites and so provide unique cleavage points (Liu *et al.*, 1995). An example of this is I-*Ceu*I which has a 26 bp restriction site that is conserved in the 23S rRNA of many eubacteria (Liu *et al.*, 1993). Alternatively, rare restriction sites can be introduced into the genome using transposons. For example Tn5 has a *Not*I site in its insertion sequence (Fonstein *et al.*, 1995).

Therefore, a mapping strategy will involve extracting intact chromosomal DNA, digestion of the chromosome at infrequent sites and separation of the

fragments by PFGE. The next challenge is to find a method of linking the fragments on the chromosomal map.

3.3. Linking fragments on genomic maps

3.3.1. Hybridization strategies

There are several methods of linking fragments on genomic maps that are based on DNA hybridization. DNA that has been restriction endonuclease digested and separated by electrophoresis is then denatured in the agarose gel and transferred to a membrane using the Southern blot technique (Southern, 1975). Large DNA molecules pose special problems in that they are difficult to transfer out of the agarose. DNA must first be cleaved using depurination or UV irradiation (Birren *et al.*, 1993). To avoid this problem, hybridization of nucleic acids can be done directly *in situ* after the gels have been denatured and completely dried (Tsao *et al*, 1983).

3.3.1.1. cross-hybridization

PFGE fragments extracted from digests prepared with one enzyme are hybridized with DNA present in PFGE gels from digests prepared with a second enzyme. Overlapping fragments hybridize to two bands in one lane thus identifying adjacent fragments on the chromosome. In principle, repetitions of this step will eventually result in all fragments being ordered. Problems with this technique include the existence of repeated sequences in the genome (eg. rRNA and insertion sequences) which lead to false positives, large probes which overlap more than two fragments so that the sections cannot be ordered and probes with very small overlaps at one end so that the overlap may be undetectable. Contamination of the probe with genomic DNA also makes interpretation of results difficult. Maps of *H.*

influenzae (Lee *et al.*, 1989), *Campylobacter jejuni* (Chang *et al.*, 1990), *Campylobacter coli* (Yan *et al.*, 1991) and *Mycoplasma fermentans* (Huang *et al.*, 1995) were constructed using cross-hybridization techniques.

3.3.1.2. hybridization with unique probes

Unique cloned fragments (genes or random clones) can be used to identify fragments from *different* enzyme digests that overlap, as opposed to using overlapping fragments which identify fragments generated by the *same* enzyme that are adjacent to each other. If enough probes exist from each area of the chromosome, an entire map can be assembled. The map of *H. ducreyi* 35000 (Hobbs *et al.*, 1996) was created using this method. Unique probes can also be used to detect the bands produced by partial digestion of the chromosomal DNA (Cole *et al.*, 1994).

3.3.1.3. hybridization with linking probes

Linking probes are cloned DNA fragments having the restriction site of the enzyme that is being used for digestion of the chromosomal DNA. Hybridization with the linking probe will reveal adjacent fragments. A complicating factor is that a method must be found to locate linking probes harboring rare restriction sites. A general strategy was employed to find *Not*I linking clones in an *Eco*RI genomic library in pBR322 for *Listeria monocytogenes* (Michel, *et al.*,1992). Pooled clones were digested with *Not*I and ligated to a Tn5 *Not*I-kamamycin-resistance cassette. After transformation in *E. coli*, when recombinant plasmids were digested with *Eco*RI and *Not*I, the result was 2 probes from each recombinant plasmid representing both ends of the insert. Hybridization of the 2 probes onto Southern blots linked adjacent fragments. Alternatively, linking clones can

be found if repeated sequences are known to exist in the genome. *Streptomyces coelicolor* has 2 copies of an insertion sequence containing an *Ase*I site so that the cloned insertion sequence served as a linking clone (Kieser *et al.*, 1992).

3.3.2. Creating new sites or deleting sites.

There are several methods of introducing rare restriction sites into the chromosmome. Lysogenic phages, transposons and integrative vectors containing restriction sites can be used. This method was used to create a physical map of *Bordetella pertussis* (Stibitz *et al.*, 1992). To define overlaps of *Spe*I and *Xba*I fragments, random insertions of a vector containing these sites were examined. An insertion of the vector resulted in disruption of *Spe*I and *Xba*I fragments, thus showing that these two fragments overlap, and the resulting sizes of the new fragment revealed the size of the overlap and the position of the insertion. Linking probes can also be used to cause the disappearance of two fragments to produce one large fragment, thus indicating linkage. To eliminate a site, methylase protection can be used (Qiang *et al.*, 1990).

3.3.3. Partial digests

Partial digests can be used, usually in conjunction with other methods, to construct genome maps (Taylor *et al*, 1992, Carlson *et al*, 1992, Michel *et al*, 1992). A band produced by a partial digest indicates that the two fragments are located together on the chromosome and can be used with a battery of probes to produce the map, such as with *Streptococcus mutans* (Okahashi *et al.*, 1990).

3.3.4. Two dimensional PFGE

The principle of two-dimensional PFGE involves digestion of the genomic DNA with the first enzyme, excision of the bands followed by digestion with the second enzyme and another PFGE step (Birren *et al.*,1993). Mapping can be accomplished with one enzyme if partial digests are compared to a complete digest when run in the second dimension, a technique used to construct the map of *Pseudomonas aeruginosa* (Romling *et al.*, 1991).

3.4. Strategy for creating the physical map of *H. ducreyi* CIP 542^T

PFGE was an ideal method for whole genome analysis of *H. ducreyi* since it is an organism for which there are no established systems of genetic exchange or collections of well characterized mutants. The physical map of the genome (Figure 1) was constructed using the following strategies: (I) single digests involving the use of four restriction endonucleases *Asc*I, *Not*I, *Sfi*I and I-*Ceu*I which cut the genome infrequently and produced fragments that could be resolved using PFGE (II) sequential reciprocal digests (III) double digests (IV) cross-hybridization whereby labeled PFGE fragments ...en from digests involving one of the four enzymes were hybridized onto digestion profiles of *Asc*I, *Not*I and *Sfi*I. In addition, select fragments from (II) and (III) were hybridized onto PFGE gels with digestion profiles of *Asc*I, *Not*I and *Sfi*I. Steps (II) and (III) were required for the determination of the relative order of restriction sites and the sizes of the overlaps. Using hybridization, I-*Ceu*I fragments were positioned on the map already created by *Asc*I, *Not*I and *Sfi*I. Sizes of overlaps were not determined by double digests for I-*Ceu*I. Overlaps of I-*Ceu*I with other fragments were approximated using information from hybridization and fragment sizes.

4. MATERIALS AND METHODS

4.1. Bacterial strains and culture conditions

H. ducreyi CIP 542^T was obtained from W. Albritton (KC1), Provincial Laboratory of Northern Alberta, and was used to create a physical map and position genes. *H. ducreyi* CIP 542 was also obtained from ATCC (#33940) and was used for comparison of restriction fragment profiles only. *H. ducreyi* was cultured for 24 h at 33^{0}C on chocolate agar (GC agar base (Difco), 1% (w/v) hemoglobin (Difco) and 1% (w/v) Isovitalex (Becton Dickson) or 0.1% (w/v) glucose, 0.01% (w/v) L-glutamine, 0.026% (w/v) L-cysteine hydrochloride) and incubated in candle jars.

4.2. Preparation of whole chromosomal DNA inserts

Bacteria were harvested by suspension in 50 mM Tris-HCl (pH8), 50 mM EDTA to approximate a No.1 McFarland turbidity standard (3 x 10^8 cells/ml). This yielded approximately 0.5 ug of DNA per gel lane (Smith *et al.*, 1990). Large clumps were allowed to settle and the remainder of the suspension was recovered. The cells were then pelleted and resuspended in Tris-HCl (pH 8), 50 mM EDTA. This step was repeated followed by one wash with 10 mM $MgCl_2$. The cells were then resuspended in 1M NaCl, 10 mM Tris HCl (pH 8) and then mixed with an equal volume of 1.5% low melting point agarose at 50^{0}C. The mixture was pipetted into a perspex plug mold (BioRad) and allowed to set. Solidified agarose plugs were placed in lysis solution (1M NaCl, 0.1M EDTA, 10 mM Tris-HCl (pH8.0), 0.5% n-lauroyl sarcosine, 1 mg/ml lysozyme, 0.1 mg/ml RNase A) and incubated at 37^{0}C in a H_2O bath with rocking for 10 h. The lysis solution was removed and blocks were incubated in digestion buffer (0.25 M EDTA, 1.0% n-lauroyl sarcosine, 0.1 mg/ml proteinase K) for 10 h at 50^{0}C in a H_2O bath with

rocking. The inserts were then exchanged at 4°C with five 50 ml changes over 2 days with 100 mM Tris-HCL (pH 8.0), 50 mM EDTA. Inserts were then allowed to equilibrate in the same solution for 7 days before restriction endonuclease digestion.

4.3. Restriction endonuclease digestion

4.3.1. single digests

All enzymes were purchased from New England BioLabs. Chromosomal DNA inserts were prepared for digestion by exchanging in 0.1 M EDTA, 10 mM Tris-HCL (pH 8.0) for 20 minutes, then equilibrating on ice for 30 minutes in the appropriate enzyme buffer and BSA. Fresh buffer was exchanged, the restriction endonuclease added, followed by further equilibration on ice for 20 minutes. Digestions were carried out for 16 to 24 h at the manufacturer's recommended temperature using 10 units of each enzyme per insert in a total volume of 200 µl.

4.3.2. sequential reciprocal digests

Inserts were digested with the appropriate endonuclease, separated by PFGE, the required fragment excised from the gel followed by digestion with the second enzyme and separation of fragments by PFGE. The same quantity of enzyme and incubation times as described above were used. A variation of this method referred to as 2-dimensional gel electrophoresis (Birren *et al.*, 1993) was also used. In the first step, DNA was digested with a single enzyme as described above and resolved by PFGE in the first dimension. The DNA-containing gel strip (i.e. one lane) was then excised and subjected to digestion with a second enzyme as follows: each gel strip was equilibrated in a hybridization bag in a 5 ml volume of manufacturer

recommended buffer overnight at 4°C. A 5 ml volume of fresh buffer was then exchanged, 50 units of enzyme added, followed by digestion for 16 to 24 h. The product was resolved in a second round of PFGE, the second dimension, using the same PFGE parameters as the first dimension.

4.3.3. double digests

Ten units of both enzymes were added at the same time to enzyme buffer. *Asc*I/*Not*I, *Not*I/*Sfi*I and *Sfi*I/*Asc*I double digests were done in the buffer recommended for *Asc*I, *Not*I and *Asc*I respectively. All double digests were performed at 37°C for 16 to 24 h.

4.4. Electrophoresis

A BioRad Chef II pulsed field electrophoresis unit was used for PFGE. Agarose gels (1.7%) were subjected to electrophoresis in 0.5 x TBE (0.045 M Tris-borate, 0.001 M EDTA) buffer at 14°C at 6 volts/cm. Run parameters used to separate fragments were 2 to 90 s for 15 h followed by 2 to 20 s for 17 h. Bacteriophage lambda concatemers having multiples of 48.5 kb (Bio-Rad) were used as a size standard. Yeast chromosomes of *S. cerevisiae* (Bio-Rad) were used to determine the size of the largest I-*Ceu*I fragment. Smaller fragments, less than 40 kb, were separated by PFGE using the run parameters 1 to 6 s for 17 h. A ladder having multiples of 5 kb (Bio-Rad) was used as a size standard. To detect fragments less than 20 kb, conventional gels were run using 0.4% agarose and electrophoresed for 24 h in 0.5 x TBE at 30 volts.

4.5. Preparation of DNA for cross-hybridization

Chromosomal DNA fragments were separated by PFGE, then excised from the gel and purified using Prep-a-Gene matrix (BioRad). DNA was then labeled with [α-^{32}P]dCTP by using a random primer labelling kit (BRL).

4.6. Preparation of probes for mapping

Inserts were excised from recombinant plasmids, purified using Prep-a-Gene matrix and radiolabelled as described for chromosomal DNA fragments 4.3.5. In some cases the plasmid-containing insert was hybridized without excision of the insert. Table1 lists the probes used.

4.7. Preparation of 16S RNA probe by PCR

The primer set P1 broad and P2 broad amplified the 16S rRNA gene (Chui et al., 1993) and were supplied by L. Chui. These primers produced a 303-bp product by PCR with all strains of *H.ducreyi*. The amplified product was excised and purified as above.

4.8. Hybridization to DNA in dried agarose gels

Hybridizations were performed directly on dried gels (Tsao *et al.*,1983). Gels were denatured using 2 x 1/2 h changes of 0.5N NaOH;150 mM NaCl and neutralized using 2 x 1/2 h changes of 500 mM Tris-HCL (pH 8);150 mM NaCl with agitation. They were placed on 2 sheets of filter paper and dried in a gel dryer to produce a thin membrane. Dried gels were pre-hybridized for 2 h in hybridization solution [5X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate);5x Denhardt's reagent (50x Denhardt's is 5%

Ficoll, 5% polyvinylpyrrolidone, 5% bovine serum albumin);0.5% SDS;100 ug/ml denatured, fragmented salmon sperm DNA], hybridized for 16 h in the same solution with probe and washed with 2 x 1/2 h changes of 0.1xSSC; 0.1% sodium dodecyl sulfate, all at 65°C. Bound probe was visualized by autoradiography. Autoradiographs were made with Kodak XAR-5 (Eastern Kodak, Rochester, N.Y.) by exposing at -70°C for 1/2 to 24 h.

4.9. Fragment nomenclature

Each fragment was named by the first two letters of the enzyme used to produce it (e.g. AscA; AsA). Likewise, fragments from double digests were given letters from the two enzymes used to produce the fragments. For example, the double digest *Asc*I-*Not*I, produced AsANoC. All I-*Ceu*I fragments begin with the designation Ce.

4.10. Preparation of figures

Computer images of gels and autoradiographs were first generated using Appligene, The Imager ™. Original images were then edited if necessary to select parts of the image and to enhance contrast of bands, using NIH Image 1.6. PowerPoint 4.0 was used to size and label figures. The genome map was drawn using Aldus IntelliDraw 2.0.

5. RESULTS

5.1. The physical genome map and genome size of *Haemophilus ducreyi* CIP 542^T

The physical genome map of *H. ducreyi* with mapped genes is shown in Figure 1, page 30 and probes are shown in Table 1, page 31. Sections 5.2, 5.3 and 5.4 comprise results that were used to construct the map.

5.1.1. genome size

Genome size of *H.ducreyi* CIP 542 was estimated to be 1771 ± 43 kb. The genome size was calculated by averaging the 21 totals obtained from 7 gels of each of 3 double digests (rather than single digests) to avoid the errors inherent in sizing large fragments. Individual fragment size (Table 2, page 33) was determined from the average of double digests from 7 PFGE gels for *Asc*I, *Not*I and *Sfi*I and 3 gels of I-*Ceu*I single digests. The total sizes of each digest (i.e. the totals at the bottom of each column in Table 2) were calculated using the average of totals of 7 gels for *Asc*I, *Not*I and *Sfi*I and 3 gels of I-*Ceu*I and 7 gels for double digests. AsI and AsJ were sized using PFGE gels with ramping over 1 to 6s for 17 h to detect small fragments.

5.1.2. ordering fragments

AsE and AsH were not ordered on the final map. Most of the other fragments on the physical map were ordered using hybridization and double digest strategies as described in Materials and Methods. The following areas require further explanation:

5.1.2.1. ordering *Asl*,J and positioning 16S and 23S rRNA on Asl

The Asl,J doublet hybridized to many areas of the map likely due to the presence of multiple copies of 16S and 23S rRNA genes, therefore this data did not conclusively position Asl and J. Other information was used to position Asl and AsJ as follows:

1) Asl and J (Figure 13) were resolved as previously described in 4.3.4. AsJ was excised and hybridized to SfA and NoA (Figure 14, fragment 21). This effectively positioned AsJ within SfA and NoA. Henceforth, all data involving hybridization of the Asl,J doublet could be used to position Asl alone.

2) CeD overlapped AsC-3 and Asl, NoD and NoH (Figure 5). This confirmed the positioning of Asl between AsC-3 and AsC-2.

3) The existence of the 18 kb double digest fragment AslNoH was confirmed by its hybridization to Asl, NoH and SfB (Figure 14, fragment 18). As in 2) above, this positioned Asl between AsC-3 and AsC-2.

4) The 16S rRNA probe (Table 5, 16S rRNA copy 6) hybridized to Asl,J doublet and SfB but not to SfA (Figure 15 and Table 5) placing 16S rRNA on Asl, not AsJ. The size of CeA and the absence of any 16S rRNA sites in the SfA fragment confirmed the positioning of 16S rRNA next to 23S rRNA on the Asl fragment.

5) The sequential reciprocal digests also aided in positioning the 50 kb fragment (Figure 6, fragment 6) generated by SfA/AscI which was identified as AsJ. It hybridized to AsI,J doublet, SfA and NoA (Figure 7, fragment 6) confirming 1) above.

6) The *SfiI/AscI* double digest 43 kb fragment (Figure 12, fragment 16) was identified as a doublet of AsI and SfBAsA by hybridization (Figure 14, fragment 16).

5.1.2.2. ordering AsD, B, J and F

AsD was positioned next to AsC-1 due to the overlap with Not F and was confirmed using double digests. The sizes of partial bands P1 (425 ± 11 kb), and P2 (274 ± 9 kb) (Figure 15, 16 and 17) added up to sizes of AsB + AsD (416 kb), and AsB + AsJ (287 kb) (Table 2, page 33), respectively. Sizes of P1 and P2 were determined from the average of sizes from 7 PFGE gels where the partial bands were visible. The order of fragments was concluded to be AsD, B, J and F.

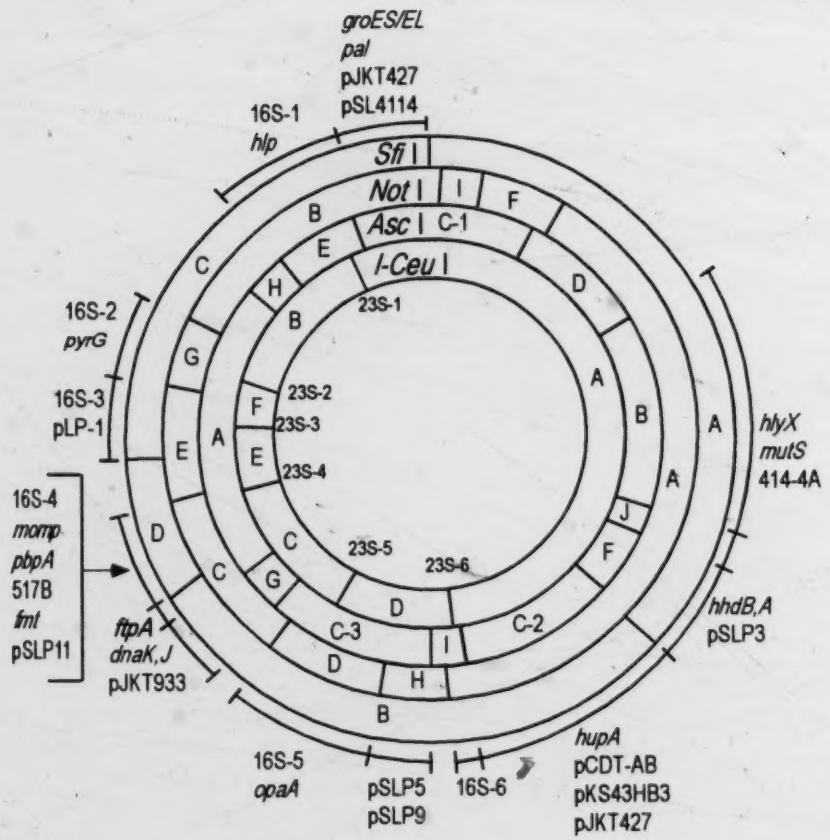


Figure 1. Physical and genetic map of *H. ducreyi* CIP 542^T. The outer to inner circles represent fragments generated by *Sfi*I, *Not*I, *Asc*I and I-*Ceu*I, respectively. Fragments AsH and AsE could not be ordered. Locations of rRNA genes and homologous *H. ducreyi* genes or DNA sequences are as indicated. Plasmid designations are listed in Table 1 for which no specific gene is known.

TABLE 1. *H. ducreyi* CIP 542^T DNA probes used in this study

PROBE	GENE DESCRIPTION	SOURCE (REFERENCE)
pPT376	*hhdB,A*, hemolysin	P.A. Totten (Totten *et al.*, 1995, Palmer *et al.*,1995, Palmer *et al.*, 1996
pPT337	*hlyX*, homolog of fumarate nitrate repressor	P.A. Totten (unpublished data)
pHD1-3	*hupA*, hemoglobin binding protein	E. Hansen (Stevens *et al.*, 1996)
pHD1-8	*pyrG*, CTP synthetase	E. Hansen, S.E. Thomas, M. Stevens (unpublished data)
pHD1-9	*fmt*, methionyl-tRNA formyltransferase	"
pHD1-12	*mutS*, DNA mismatch repair protein	"
pHD1-13	*pbpA*, penicillin binding protein 2	"
pSLP3	bind plasminogen	"
pSLP5	"	"
pSLP9	"	"
pSLP11	"	"
pSL705	*opaA*, cell envelope protein associated with colony opacity in *H. influenzae*	"
pSL4114	involved in expression of a surface-exposed LOS epitope	"
pCDT-AB	cytolethal distending toxin-like genes	"
pJKT427	UDP-glucose epimerase, an enzyme involved in the biosynthesis of LOS	"
pJKT600	*momp*, 5' half of gene encoding major outer	"

	membrane protein (OmpA homologue)	
pJKT933	protein released by *H. ducreyi* into culture supernatant	"
pKS-43HB3.1	protein with 44% identity to lipid A disaccharide synthase of *E.coli*	"
p414-A	cell envelope antigen	"
p517B	protein with leader peptide	"
pHD18	*pal*, 18K OMP (PAL homologue)	S. Spinola (Spinola *et al.*, 1996)
pHD24	*ftpA*, 24K pilin monomer, (fine tangled pili)	S. Spinola (Brentjens, *et al.*, 1996)
pHD28	*hlp*, 28K outer membrane lipoprotein	S. Spinola (Hiltke *et al.*, 1994)
pHD40	*momp*, C-terminal half of the major outer membrane protein (Omp A homologue)	S. Spinola (Spinola *et al.*, 1993, Hiltke *et al.*, 1995)
pLP-1	2 ribosomal protein genes	L. Parsons (unpublished data)
pLP8BS	*groES/EL*, heat-shock proteins,	L. Parsons (Parsons *et al.*, 1992)
pLP14	*dnaK,J* heat shock proteins	L. Parsons (Parsons *et al.*, 1995)
pKLP104	*hhdB,A*, majority of hemolysin operon	B. Munson (Totten *et al.*, 1995, Palmer *et al.*, 1995, Palmer *et al.*, 1996)
PCR product	16S rRNA gene from *H. ducreyi*	L. Chui (Chui *et al.*, 1993)

TABLE 2. Restriction endonuclease fragments of the chromosome of *H.ducreyi* produced by *Asc*I, *Not*I, *Sfi*I and I-*Ceu*I digestion

FRAGMENT	SIZE OF FRAGMENTS (kb ± standard deviation)						
	*Asc*I	*Not*I	*Sfi*I	*I-Ceu*I	*Asc*I-*Not*I	*Not*I-*Sfi*I	*Sfi*I-*Asc*I
A	373±14	707±30	620±27	1000±0	251±4 (AsB)	495±20 (SfANoA)	251±4 (AsB)
B	251±4	313±11	513±33	258±2	199±4 (AsC-2)	306±9 (SfCNoB)	204*±7 (SfCAsA)
C	199, 199, 199 ±4	197±5	435±23	235±5	150±4 (AsDNoA)	210±6 (SfBNoA)	199±4 (SfBAsC-2 + AsC-3)
D	165±6	131±4	137±7	128±2	131±4 (NoD)	131±4 (NoD)	165±6 (AsD)
E	105±3	123±4		109±3	123±4 (NoE)	119±5 (SfBNoC)	137±7 (SfD)
F	75±3	79±2		65±0	116±4 (AsANoC)	83±3 (SfDNoC)	110*±5 (SfAAsC-1)
G	62±3	75±3			105±3 (AsE)	79±2 (NoF)	105±3 (AsE)
H	56±2	64±4			94*±2 (AsC-1NoB)	74±8 (SfCNoE + NoG)	84±5 (SfCAsC-1)

						+ NoG)	
I	43±3	47±2			75[a]±3 (AsF + NoG)	64±4 (NoH)	75±3 (AsF)
J	36[b]±0				69[a]±2 (AsC-1NoF + AsANoB)	55±3 (SfDNoE)	62±3 (AsG)
					62±3 (AsG)	47±2 (NoI)	55±3 (AsH)
					55[a]±3 (AsH + AsC-3NoH)	12[d]±0 (SfANoB)	43[a]±3(AsI + SfBAsA)
					47[a]±2 (NoI)		36[d]±0 (AsJ)
					36 [a,d]±0 (AsJ)		9[d]±0 (SfAAsC-2)
					25 [a,c]±5 (AsINoA)		
					18[a,c]±0 (AsINoH + AsDNoF + AsC-3NoC)		
Total	1761±44[e]	1736±53[e]	1706±80[e]	1796±10[f]	1788±37[e]	1744±36[e]	1774±36[e]

[a]cross-hybridized

[b]sized from 1-6s PFGE, AsI and AsJ appear as a doublet using standard PFGE conditions (Figure 2) and as resolved fragments using switch times of 1-6s (Figure 13)

[c]sized from 1-6s PFGE, fragment not visible using standard PFGE conditions (Figure 10) but is visible using switch times of 1-6s (Figure 13)

[d]sized from 1-6 s PFGE, band not visible using standard PFGE conditions (Figure 2) but is visible using switch times of 1-6s PFGE (Figure 13)

[e]average of totals of 7 gels

[f]average of totals of 3 gels

5.2. Single digest gels and cross-hybridization of single digest fragments

Figures 2 and 3 are PFGE gels of *H.ducreyi* chromosomal DNA digested with *Asc*I, *Not*I, *Sfi*I and I-*Ceu*I. Restriction fragment sizes obtained from the single digests are listed in Table 2, page 33. These enzymes produced 12, 9, 4 and 6 fragments, respectively. *Asc*I produced one triplet, AsC. The smallest *Asc*I fragments I and J appeared as a single band under standard PFGE conditions (Figure 2) but were resolved by PFGE by ramping over 1 to 6s for 17 h (Figure 13). No bands less than 20 kb were detected for single digests involving *Asc*I, *Not*I and *Sfi*I.

Single digest fragments were cross-hybridized and results are listed in Table 3. Early in the project NoDE and NoFGH were not adequately resolved for hybridization data to be interpreted and these instances are indicated in Table 3. Later gels resolved NoDE and NoFGH fragments so that they could be individually exised and hybridized, as shown in Table 3. Figure 4 and Figure 5 present the series of autoradiographs which positioned the 9 fragments generated by *Not*I and the 6 fragments generated by I-*Ceu*I on the map. NoA and CeA were large fragments and did not hybridize to the small AsF and AsJ fragments. NoF hybridized poorly to AsD presumably due to the small overlap. This overlap was identified later in double digest experiments.

Overlapping fragments were identified when hybridization occurred to more than one band in one lane. Only the brightest bands were considered when interpretation was made difficult by nonspecific hybridization presumably due to repeated sequences existing in the genome or contamination of the

probe fragment with DNA from other fragments. In cases where the map suggested that small overlaps were occurring or that a large fragment was hybridizing to a small fragment, bands were not expected to be bright due to poor hybridization.

Successful single digests allowed an estimation of genome size and estimates for all 4 digests were similar. Resolution of all fragments by PFGE allowed a preliminary map to be constructed by excision and cross-hybridization of fragments. However, the problems of hybridization discussed above indicated that not all overlapping fragments could be detected and non-specific hybridization may have resulted in some inaccuracies in the map. Therefore, other methods were necessary to confirm the accuracy of the map.

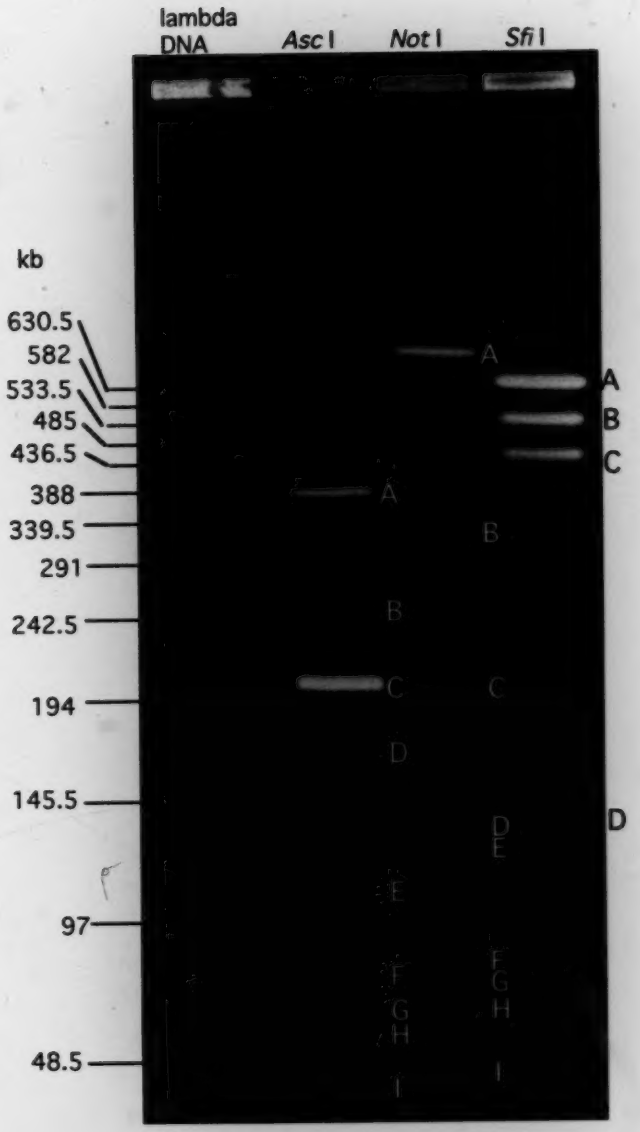


Figure 2. PFGE separation of single digestion fragments produced by *Asc*I, *Not*I and *Sfi*I digestion of *H. ducreyi* CIP 542 chromosomal DNA. The enzymes used to generate each lane are identified at the top of the gel. The fragment designations are given beside each band. Sizes and band positions of lambda DNA concatemers are given on the left side of the panel.

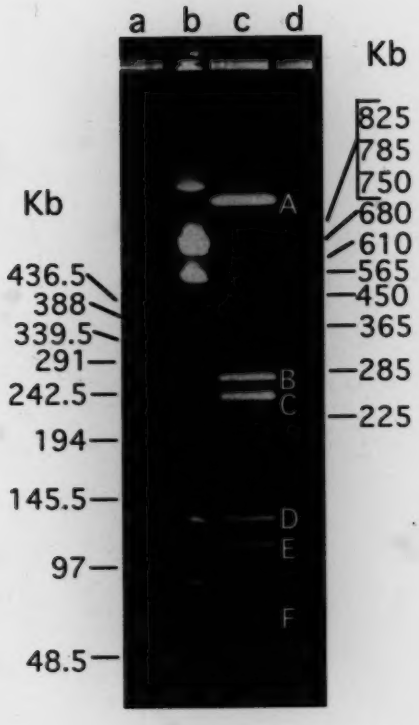


Figure 3. PFGE separation of I-*Ceu*I digestion fragments of *H. ducreyi* CIP 542 chromosomal DNA. Lane (a), lambda DNA concatemers; lane (b), control *E.coli* W3110 chromosomal DNA supplied with enzyme. Sizes of fragments are 45, 90, 120, 530, 670 and 700 (unresolved in this gel), and 2470 kb; lane (c) *H. ducreyi* CIP 542 I-*Ceu*I digest; lane (d), yeast chromosome *S. cerevisiae* size standard. The fragment designation of single digests generated using I-*Ceu*I are given beside each band. The sizes of DNA markers in lane (a) and (d) are given on the left and right side respectively of the panel.

TABLE 3. Cross-hybridization of restriction fragments generated by digestions with *Asc*I, *Not*I, *Sfi*I and I-*Ceu*I

PROBE	*Asc*I profile	*Not*I profile	*Sfi*I profile
AsA	A	B,C, (D or E; F, G or H)[a]	B,C,D
AsB	B	A	A
AsC	C	A,B,C,D,F,H,I	ND[b]
AsD	D	A	A
AsE	E	B	C
AsF	F	A	A
AsG	G	C	B
AsH	H	B	C
AsI,Jd[c]	I,Jd	A (weak)	B
AsJ[d]	I,Jd	A	A
NoA	B,C	A	A,B
NoB	A,C,E,H	B	C
NoC	A,C,G,	C	B,D
NoD	C	D	B
NoE	A	E	C,D
NoF	C	F	A
NoG	A	G	C
NoH	C	H	B
NoI	C	I	A
SfA	ND	A,B,F,I	A
SfB	ND	A,C, (D or E; F, G or H)[a]	B
SfC	ND	B, (D or E; F, G or H)[a]	C
SfD	ND	C,(D,E)[a]	D

CeA	B,C,D,F	A,B,F,I	A,B,C
CeB	A,E,H	B,G	C
CeC	A,C,G	C,(D.E)[a]	B,D
CeD	C,(I,J)d	D,H	B
CeE	A	C,E	C,D
CeF	A	E,G	C

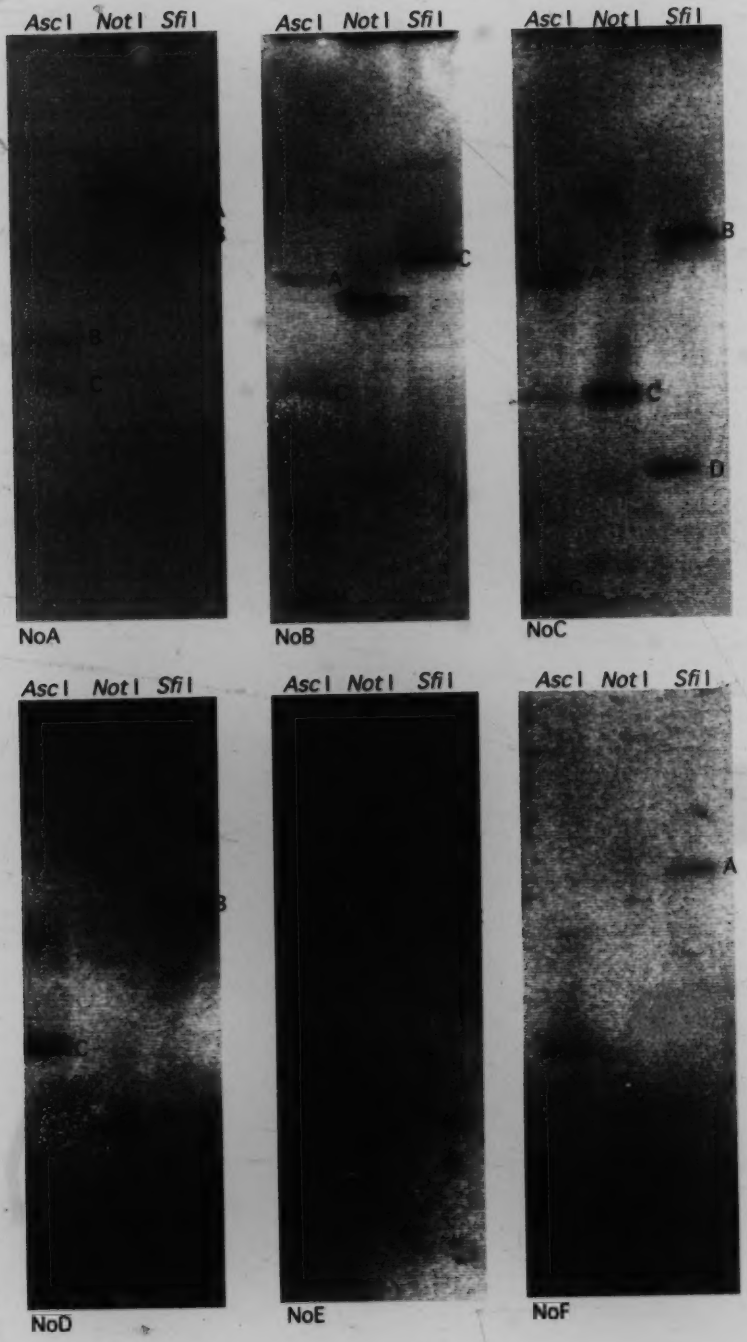
[a]DE,FGH too closely spaced to interpret hybridization

[b]not done

[c]doublet

[d]fragment excised from 1-6s PFGE

Figure 4. Autoradiographs of the cross-hybridization of fragments generated by digestion of *H. ducreyi* genomic DNA with *Not*I using gels with restriction profiles of *Asc*I, *Not*I and *Sfi*I. The enzymes used to generate each lane are identified at the top of each autoradiograph. The probe used is identified at the bottom of each autoradiograph. Bands to which the probes hybridized are labelled.


Asc I Not I Sfi I
B
C
NoA
Asc I Not I Sfi I
C
A
NoB
Asc I Not I Sfi I
B
C
D
NoC
Asc I Not I Sfi I
B
C
NoD
Asc I Not I Sfi I
NoE
Asc I Not I Sfi I
A
NoF

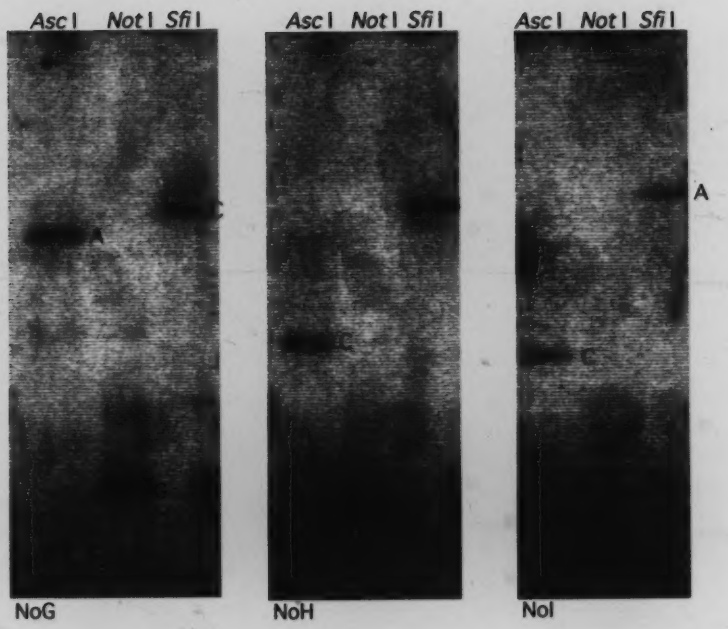
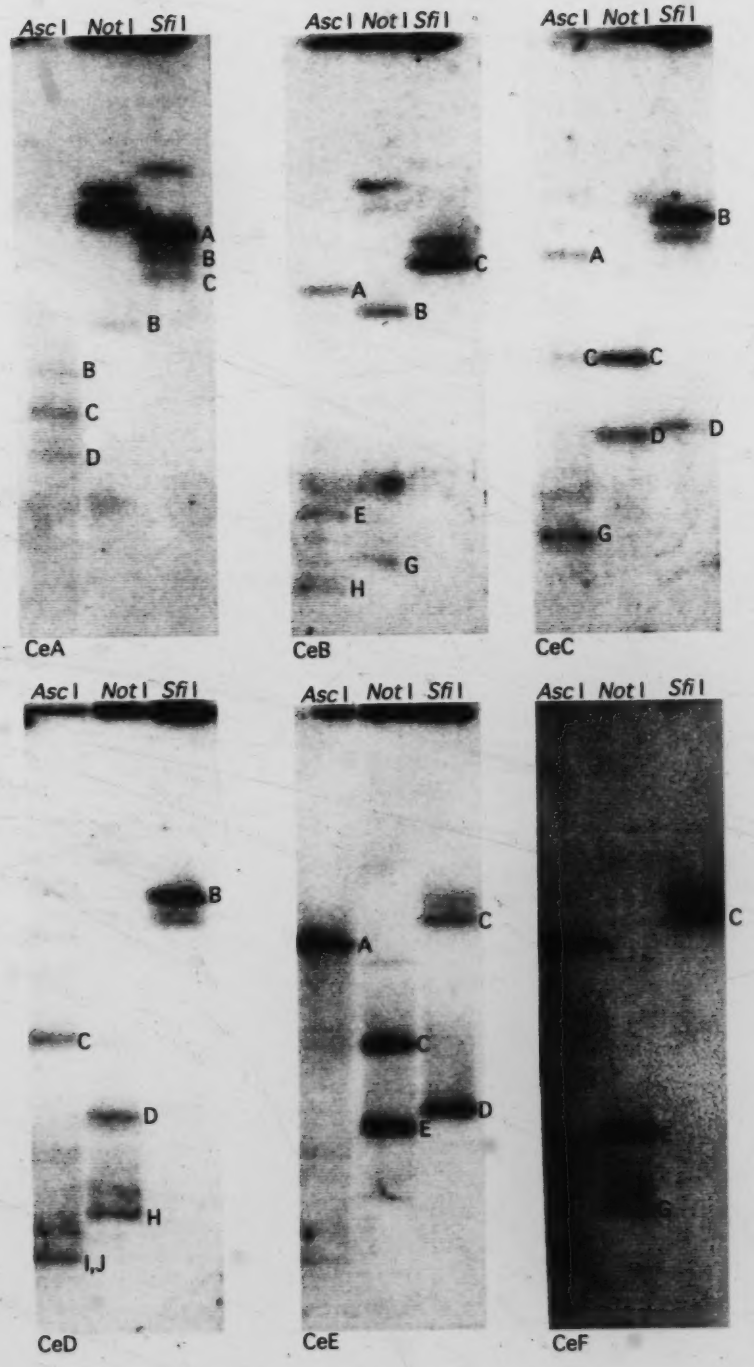

Asc I
Not I
Sfi I
A
C
NoG
Asc I
Not I
Sfi I
C
NoH
Asc I
Not I
Sfi I
A
C
NoI

Figure 5. Autoradiographs of the cross-hybridization of fragments generated by digestion of *H. ducreyi* genomic DNA with I-*Ceu*I using gels with restriction profiles of *Asc*I, *Not*I and *Sfi*I. The enzymes used to generate each lane are identified at the top of each autoradiograph. The probes used are identified at the bottom of each autoradiograph. Bands to which the probes hybridized are labelled.


Asc I Not I Sfi I
A
B
C
B
B
C
D
CeA
Asc I Not I Sfi I
C
A
B
E
G
H
CeB
Asc I Not I Sfi I
B
C
C
D
D
G
CeC
Asc I Not I Sfi I
B
C
D
H
I,J
CeD
Asc I Not I Sfi I
C
A
C
D
E
CeE
Asc I Not I Sfi I
C
G
CeF

5.3. Sequential reciprocal digests gels and cross-hybridization of sequential reciprocal digest fragments

Figure 6 shows the gel produced by PFGE with sequential reciprocal digests involving fragments SfA, B, C and D, digested with *Asc*I and AsC and H digested with *Sfi*I. Select fragments were cross-hybridized as indicated in Figure 6 to confirm their identity. Figure 7 shows autoradiographs of the cross-hybridization experiments. Figure 8 shows digests of NoA, B, C, D, E and F digested with *Asc*I. All fragments produced by sequential reciprocal digest experiments are listed in Table 4. Figure 9 shows sequential reciprocal digests of NoA, B, C, D, E, FG, and H digested with *Sfi*I using a 2-dimensional PFGE gel.

The sequential reciprocal digests also aided in positioning the 50 kb fragment (Figure 6, fragment 6) generated by SfA/AscI. It hybridized to AsI+J doublet, NoA and SfA. As discussed in Section 5.1, AsI was positioned separate from AsJ on the SfB fragment.

5.3.1. positioning of the AsC triplet

The results presented in Figure 6 and 7 identified the position of the AsC triplet. By digesting all four *Sfi*I bands separately with *Asc*I and the AsC triplet with *Sfi*I (Figure 6) and then looking for bands of the same size (i.e. the overlapping fragments), the exact positions of AsC-1, 2 and 3 and sizes of overlaps were established. Digests SfA/*Asc*I and AsC/*Sfi*I produced a common fragment of approximately 125 kb, the size of the overlap of AsC-1 with SfA. Likewise, digests SfC/*Asc*I and AsC/*Sfi*I both produced a 100 kb band (Figure 6, fragments 3 and 4) the size of the overlap of AsC-1 with

SfC. The position of this fragment was further proven by hybridization of the fragment generated by the separate digests (Figure 7, fragments 3 and 4) indicating they hybridized to the same position and are therefore the same fragment. Similarily, both digests SfB/*Asc*I and AsC/*Sfi*I generated a band of aproximately 200 kb (Figure 6, fragments 1 and 2) which hybridized to NoA, C, D and H and SfB (Figure 7, fragments 1 and 2). The intensity of the band also indicated that it was a doublet. AsC-2 and 3 were therefore positioned within the SfB fragment.

5.3.2. discussion of the sequential reciprocal digest method

One method of performing sequential reciprocal digests was to excise bands produced by digestion with one enzyme followed by digestion with a second enzyme. This identifies restriction sites of a second enzyme within a segment of the chromosome generated by the first enzyme. If the sizes of bands generated can be compared to cross-hybridization data from hybridization of single digest fragments, then each band can be identified and the exact sizes of overlaps can be established. This method was successfully used for *Treponema denticola* (MacDougall *et al.*, 1995) and *Streptococcus thermophilus* (Roussel *et al.*, 1994). This method is more informative than a double digest which will confirm bands having internal restriction sites but will not easily identify sizes of overlaps.

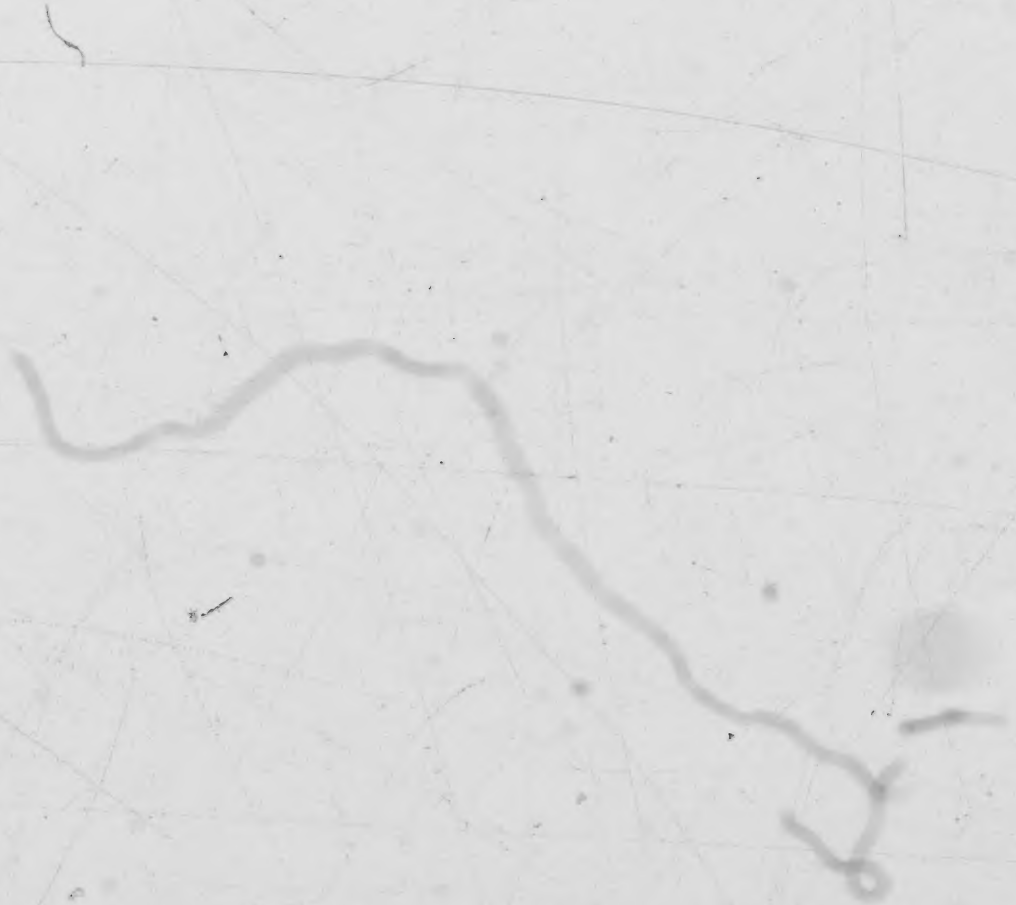
There were problems with the use of sequential reciprocal digests together with data from hybridization of single digests. As mentioned above, errors occur in interpreting hybridization patterns. This problem was more exaggerated if the band in the sequential digest gel was a section of an overlapping fragment and could be mistaken for a small uncut whole

fragment. Further hybridization of bands from the sequential digest gel was needed to confirm the identity of some fragments.

Technical difficulties occured when performing sequential reciprocal digests. Bands must be identified by ethidium bromide staining before excision from the gel and the staining may have resulted in poor digestion with the second enzyme. It was difficult to identify those bands which were partially digested and those which were digested completely. Two samples can be run side by side, one lane stained and nicked to mark bands for excision, positioned back on the gel and used as a guide for excision of the unstained band (Birren *et al.*, 1993). Both stained and unstained bands were used in this project and both gave similar results. In addition, excised bands were loaded onto PFGE gels without either trimming off excess agarose or purification of DNA to avoid damaging large pieces of DNA. Therefore, the precise location of the origin was unclear resulting in inaccurate sizing of the bands. This could also lead to misidentification of a band.

Results obtained from the 2-dimensional gel electrophoresis experiments (Figure 9) confirmed the number of internal *Sfi*I restriction sites within each *Not*I fragment. NoCSfB and NoCSfD appeared as one long vertical line which may have been the two fragments together. NoESfD, a 55 kb fragment was missing and may have run off the gel. The same problem existed as mentioned above in that sizing of bands was not accurate due to the uncertainty of the origin of the DNA, a problem also noted by MacDougall *et al* in construction of the map of *Treponema denticola* (MacDougall *et al.*, 1995). The bands were assessed relative to each other rather than by absolute size. In addition, the bands were vertically elongated in contrast to

the horizontal bands of the lambda DNA marker. A large amount of enzyme was required to perform the 2-dimensional electrophoresis, approximately 10 times as much as the sequential double digest in which fragments were excised individually.



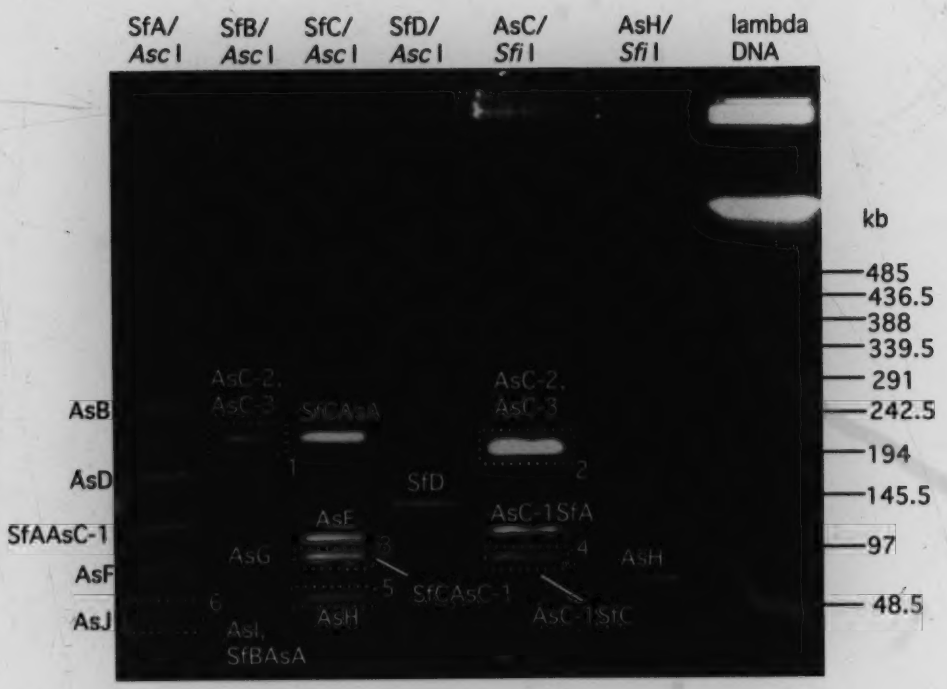
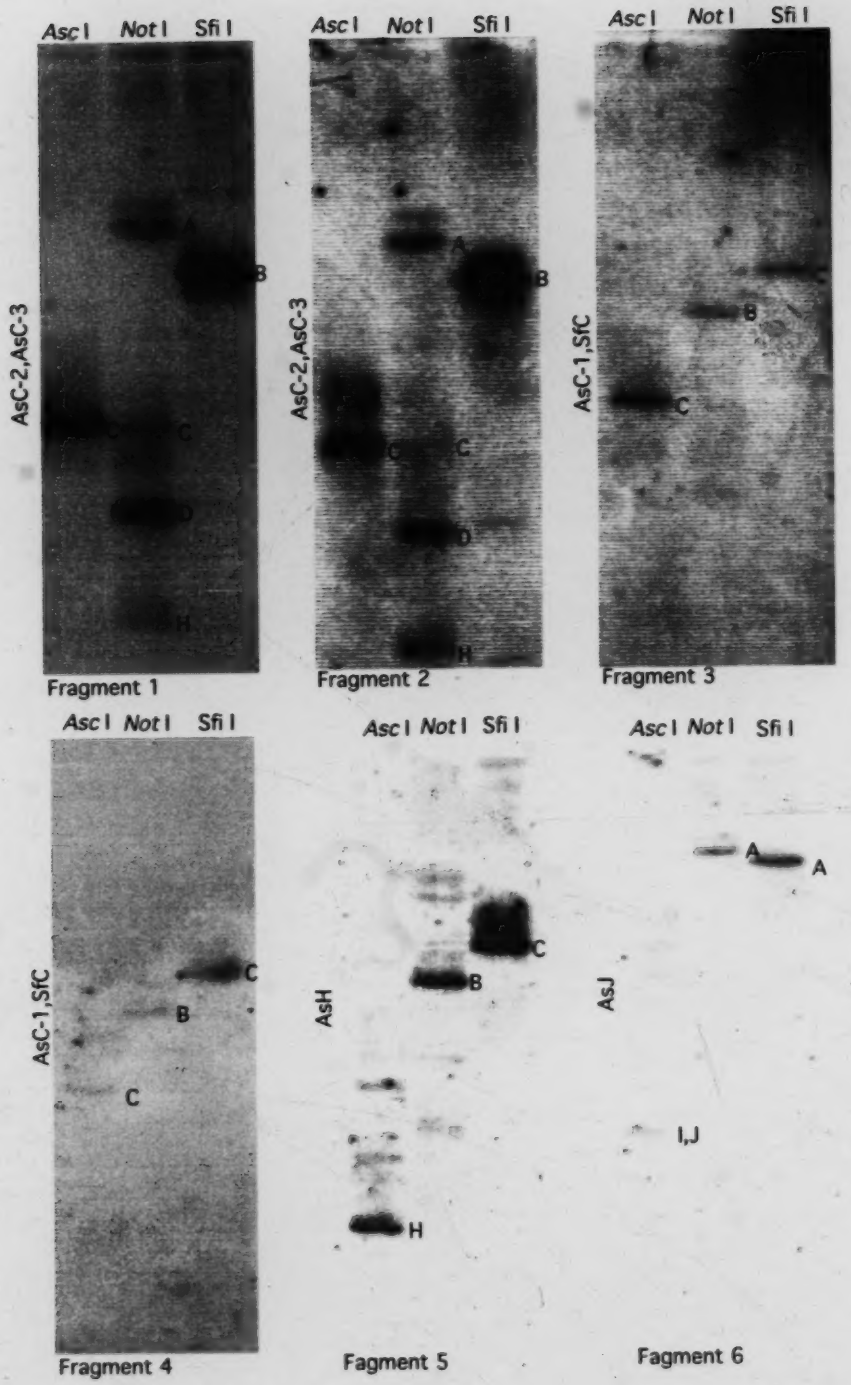


Figure 6. PFGE gel of sequential reciprocal digests of fragments SfA, B, C and D digested with *Asc*I and fragments AsA, C and H digested with *Sfi*I. Each lane is labelled at the top with the band excised followed by the second digestion enzyme. The numbered dotted rectangles indicate fragments that were cross-hybridized and correspond to the fragment number on the bottom of each autoradiograph in Figure 7.

Figure 7. Autoradiographs of cross-hybridized fragments excised from sequential reciprocal digests PFGE gels. The enzymes used to generate each lane are identified at the top of each autoradiograph. The probes corresponding to the numbered dotted rectangles in Figure 6 are identified at the bottom of each autoradiograph. The probes are also identified by fragment designation on the left side of each autoradiograph.


Asc I Not I Sfi I
AsC-2,AsC-3
A
B
C
D
H
Fragment 1
Asc I Not I Sfi I
AsC-2,AsC-3
A
B
C
C
D
H
Fragment 2
Asc I Not I Sfi I
AsC-1,SfC
B
C
Fragment 3
Asc I Not I Sfi I
AsC-1,SfC
C
B
C
Fragment 4
Asc I Not I Sfi I
AsH
C
B
H
Fagment 5
Asc I Not I Sfi I
AsJ
A
A
I,J
Fagment 6

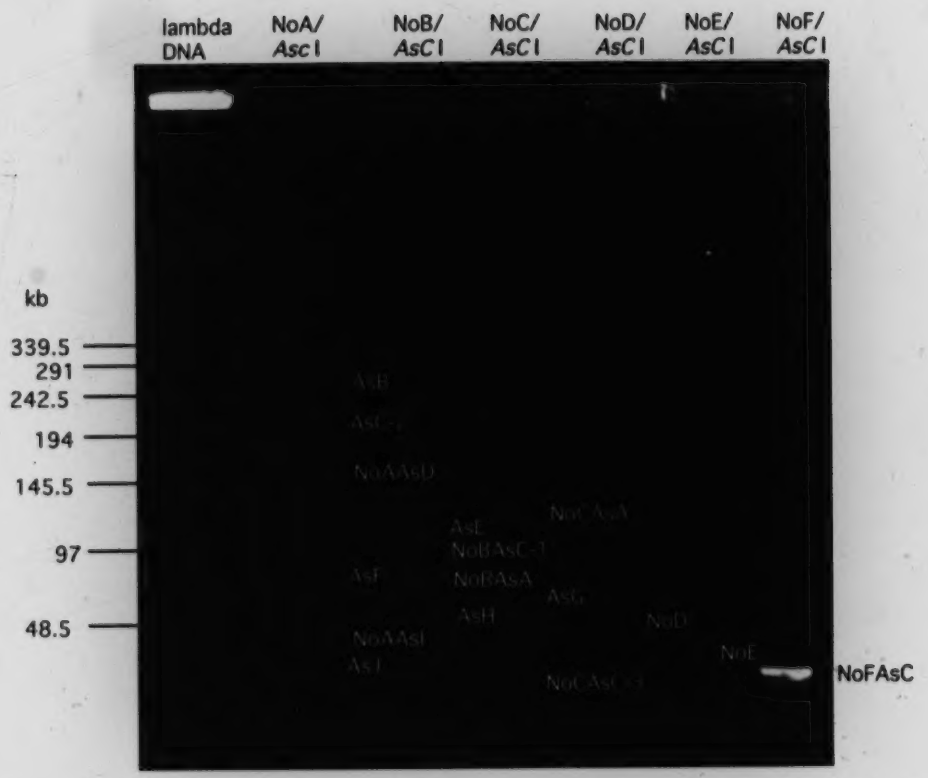


Figure 8. PFGE gel of sequential reciprocal digests of fragments NoA, B, C, D, E and F digested with *Asc*I. Each lane is labelled at the top with the band excised followed by the second digestion enzyme.

TABLE 4. Sequential double digests

First/ second enzyme	Restriction fragment excised	Fragment sizes(kb)	Fragment
SfiI/NotI	SfA	550,75,35	SfANoA,NoF,NoI
	SfB	225d[a],137,66	SfBNoA + SfBNoC,NoD,NoH
SfiI/AscI	SfA	260,175, 127,82,48[b]	AsB,AsD,SfAAsC-1, AsF,AsJ
	SfB	200d[b],69, 48d	AsC-2 + AsC-3,AsG,AsI + SfBAsA
	SfC	190,110,91[b],62[b]	SfCAsA,AsE, SfCAsC-1, AsH
	SfD	145	SfD
NotI/SfiI	NoA	540,200	NoASfA,NoASfB
	NoB	330[c]	NoBSfC
	NoC	137,75	NoCSfB,NoCSfD
	NoDE	145,80,55	NoD, NoESfD, NoESfC
	NoFGHI	80,62, 58d	NoF,NoG,NoH + NoI
NotI/ AscI	NoA	268,215,160,77,43 ,35	AsB, AsC-2, NoAAsD, AsF,NoAAsI,AsJ
	NoB	110,99,70,58	AsE,NoBAsC-1,NoBAsA, AsH
	NoC	118,63,30	NoCAsA,AsG,NoCAsC-3
	NoDE	130d	NoD + NoE
	NoE	126	NoE
	NoF	68[d]	NoFAsC-1
	NoG	70	NoG
	NoH	52,28[e]	NoHAsC-3,NoHAsI
	NoI	45	NoI
AscI/SfiI	AsA	222[b],146[b],45[b]	AsASfC,SfD,AsASfB
	AsC	212d[b],123,97[b]	AsC-2 + AsC-3,

			AsC-1SfA, AsC-1SfC
	AsH	56	AsH
AscI/ NotI	AsA	124d,80,74	AsANoC + NoE,NoG,AsANoB
	AsB	270	AsB
	AsD	150[d]	AsDNoA
	AsE	108[f]	AsE
	AsF	84[f]	AsF
	AsG	76[f]	AsG
	AsH	66[f]	AsH
	AsI + J	48.5[g]	AsJ

[a]d refers to doublet

[b]fragment cross-hybridized onto gels having single digests of *Asc*I, *Not*I and *Sfi*I

[c]12 kb NoBSfA assumed to have run off gel

[d]18 kb AsDNoF assumed to have run off gel

[e]fragment below 48.5 kb marker therefore sizing inaccurate

[f]precise origin of DNA not known therefore sizing inaccurate (see materials and methods)

[g]25 kb AsINoA and 18 kb AsINoH assumed to have run off gel

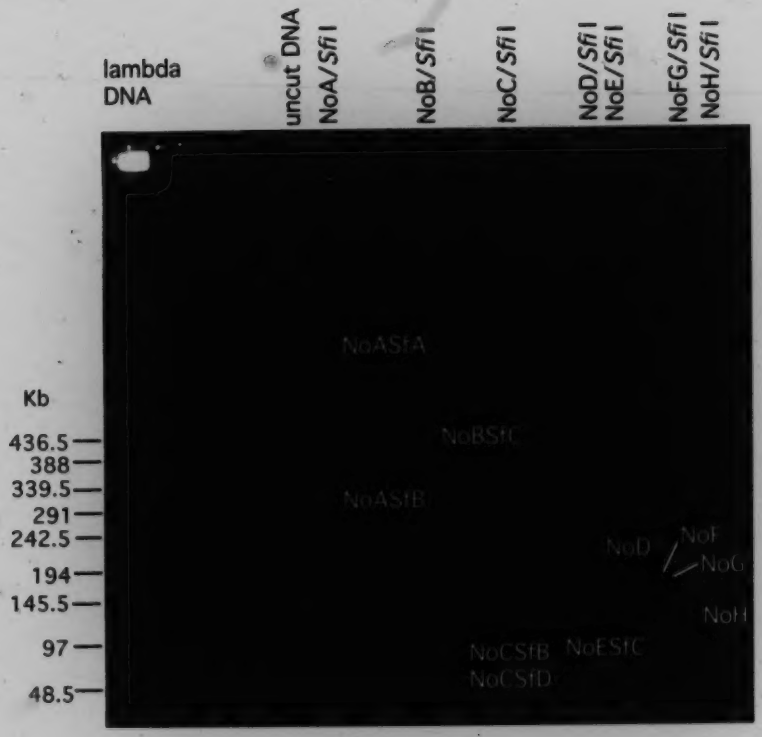


Figure 9. Two-dimensional PFGE gel of sequential reciprocal digests of NoA, B, C, D, E, FG, and H digested with *Sfi*I.

5.4. Double-digest gels and cross-hybridization of double-digest fragments

Figures 10,11,12 and 13 show PFGE gels of double digests of genomic DNA. Numbered fragments were cross-hybridized (Figure 14). Table 2, page 33 lists fragments generated by the double digests which were used to make final calculations of genome size. Double digests of *Not*I/*Sfi*I and *Sfi*I/*Asc*I produced 12 kb and 9 kb fragments (Figure 13, fragments 19 and 20) and were resolved by both PFGE and conventional gel electrophoresis. The *Asc*I/*Not*I double digest produced 25 kb (fragment 17) and 18 kb fragments (fragment 18). The 18 kb fragment proved to be a triplet after cross-hybridization (Figure 14, fragment 18). Figure 14, fragment 16 showed that the 43 kb fragment generated by *Sfi*I/*AsC*I was a doublet of AsI and SfBAsA.

Many of the autoradiographs in Figure 14 illustrate the problem of non-specific hybridization, in particular, fragment 17, AsINoA. This is likely due to the presence of 16S and 23S rRNA sequence in this region.

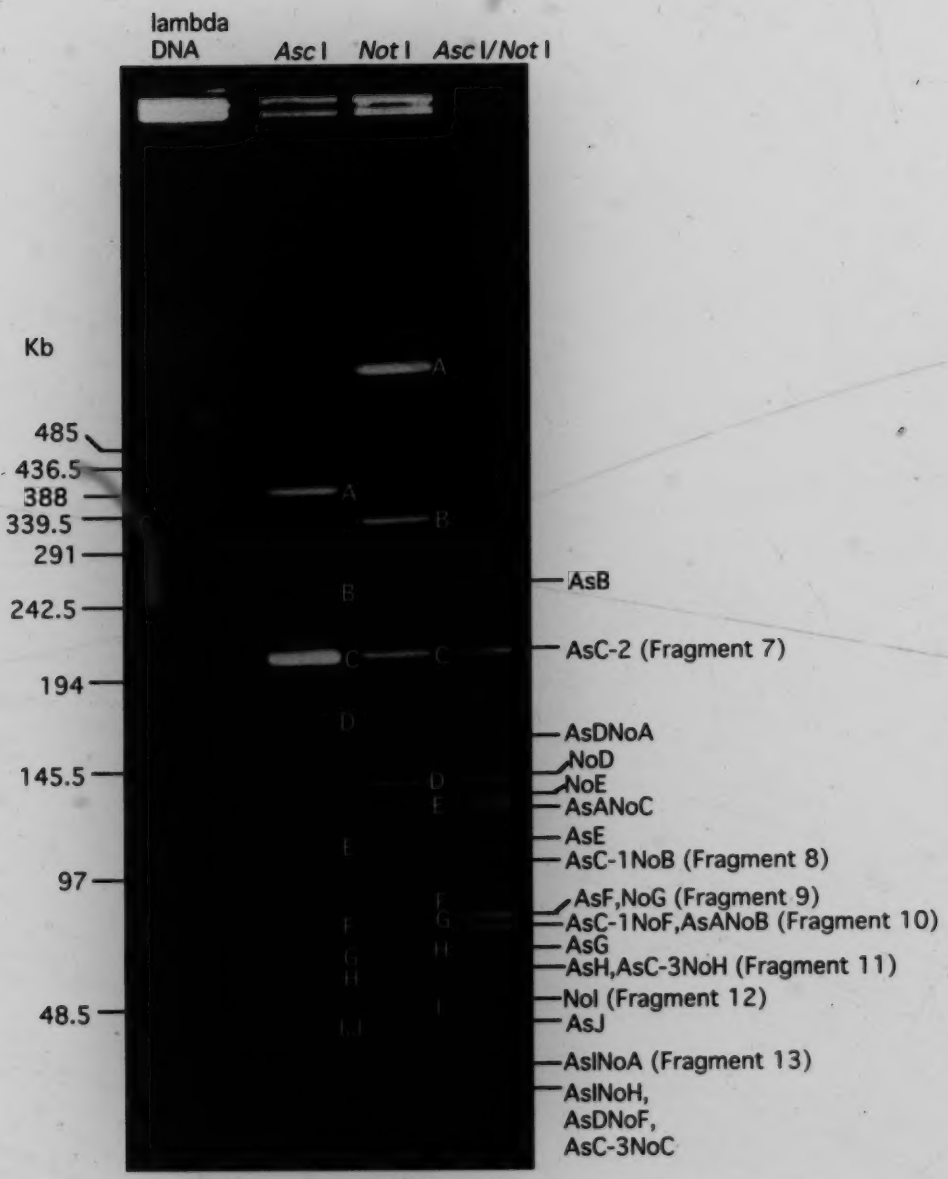


Figure 10. PFGE gel of double digests of genomic DNA generated by *AscI/NotI*

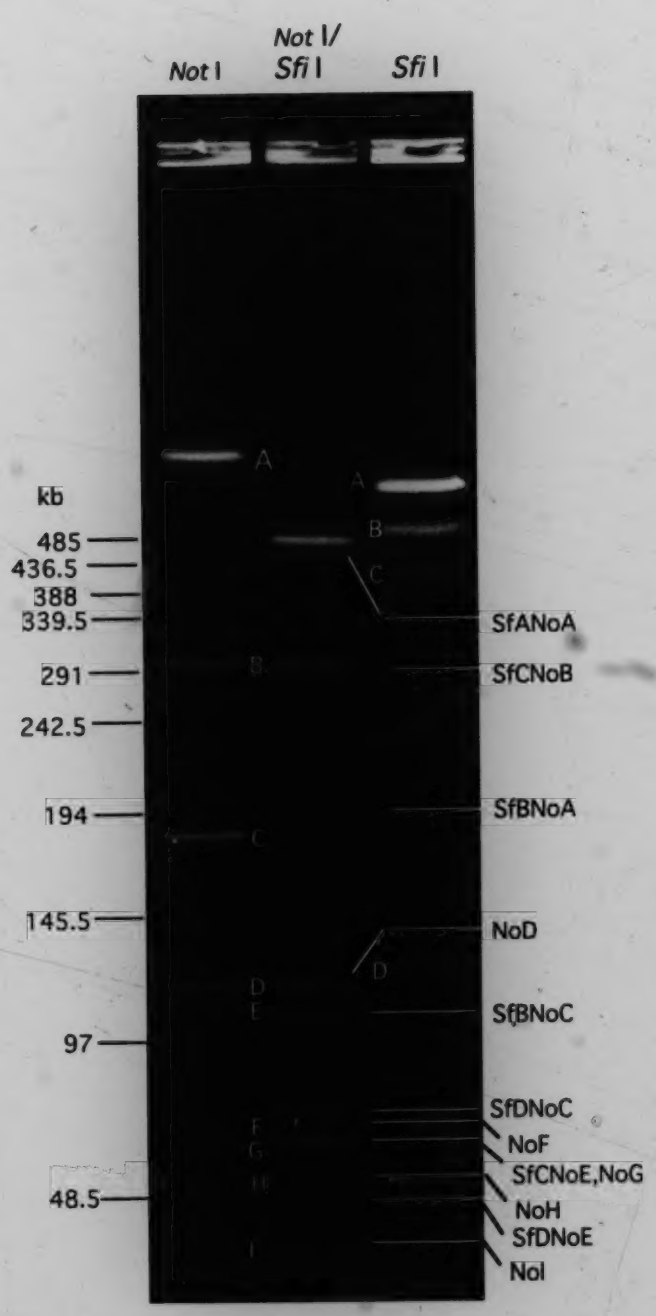


Figure 11. PFGE gel of double digests of genomic DNA generated by *NotI/SfiI*.

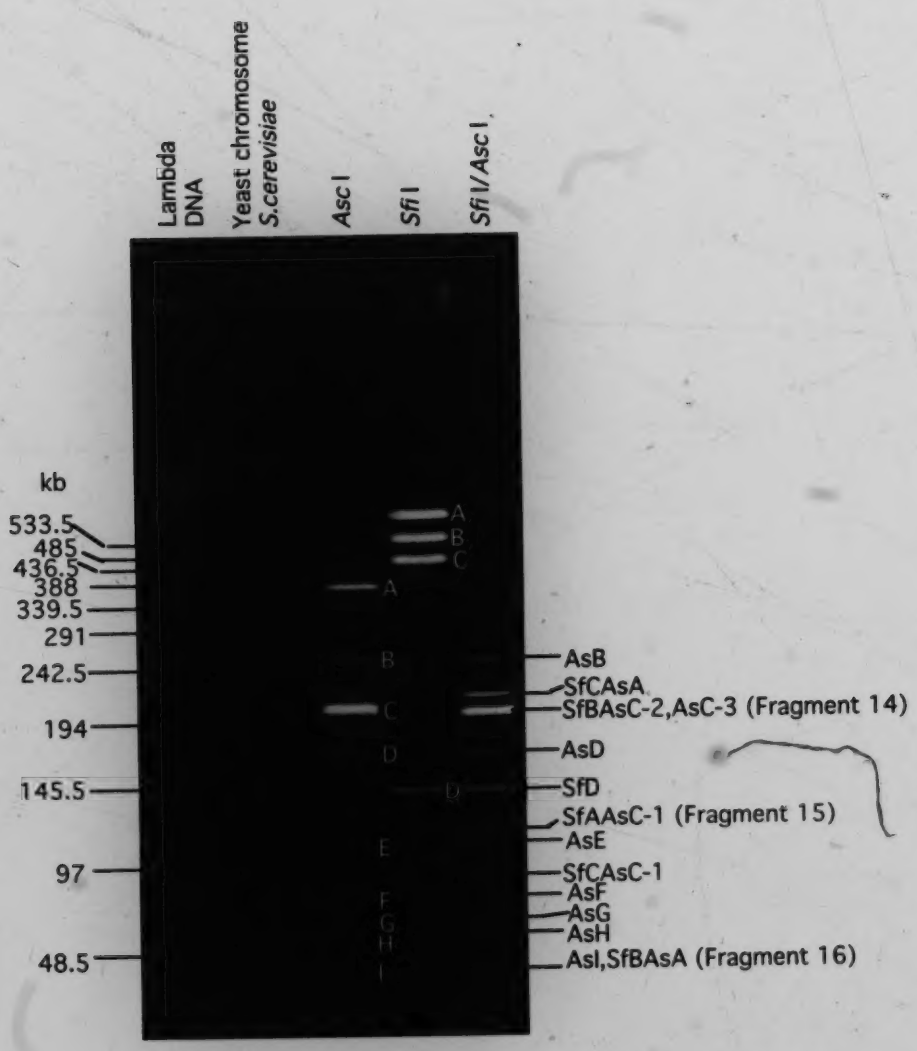


Figure 12. PFGE gel of double digests of genomic DNA generated by *SfiI/AscI*. Sizes of the yeast chromosome *S. cerevisiae* are as follows: 225, 285, 365, 450, 565, 610, 680 and (750, 785, 825 unresolved) kb.

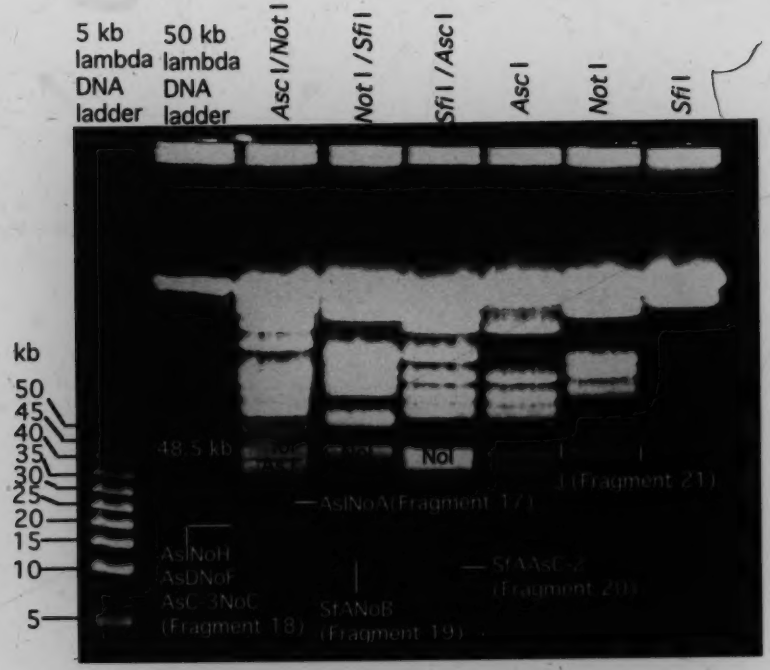
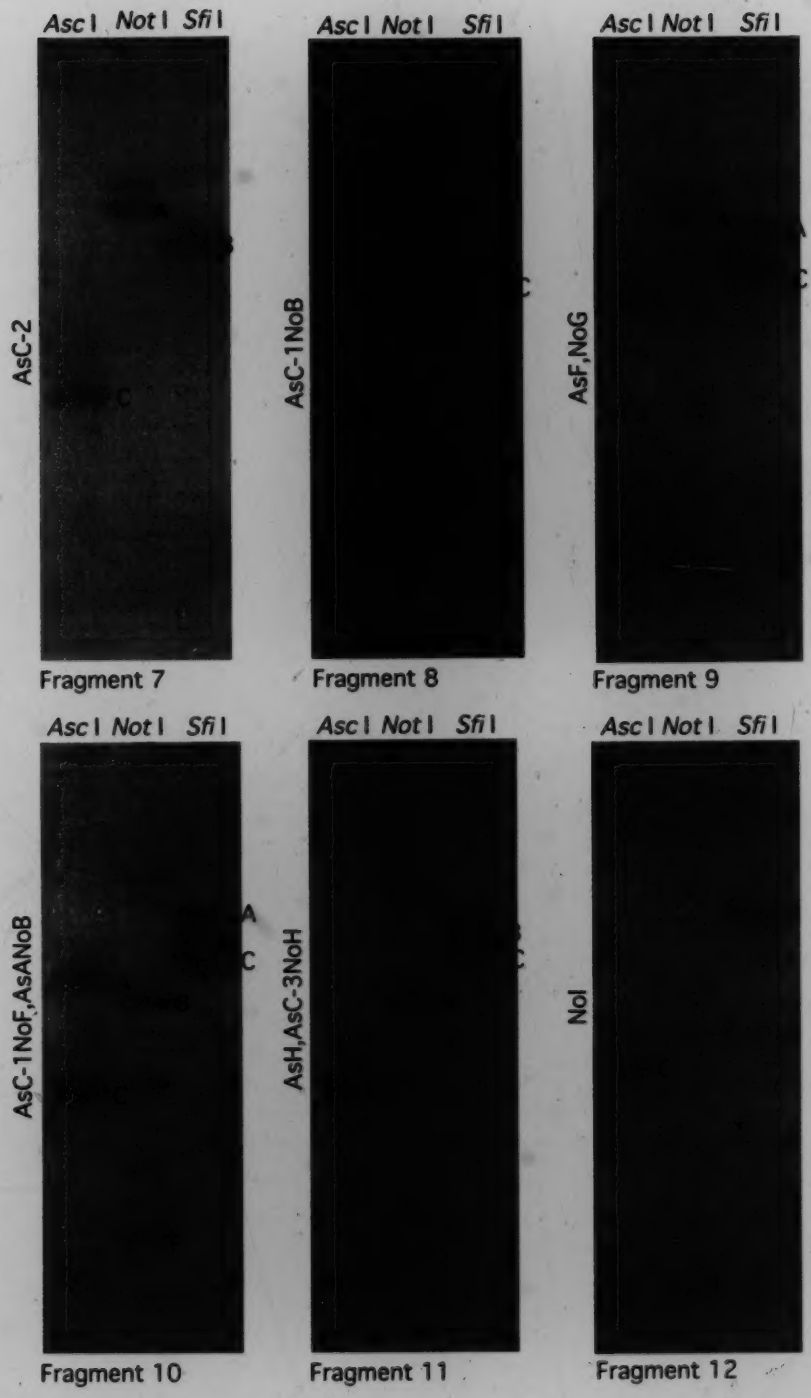
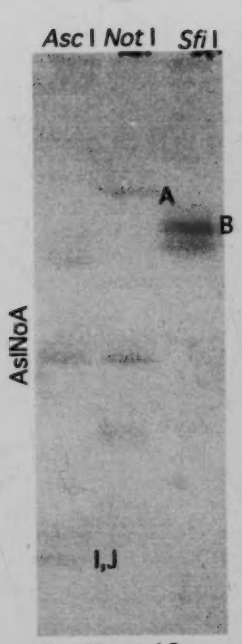


Figure 13. PFGE gel of double and single digests of genomic DNA generated by *Asc*I/*Not*I, *Not*I/*Sfi*I, *Sfi*I/*Asc*I, *Asc*I, *Not*I and *Sfi*I, with ramping over 1-6s for 17h to detect fragments less than 40 kb in size.

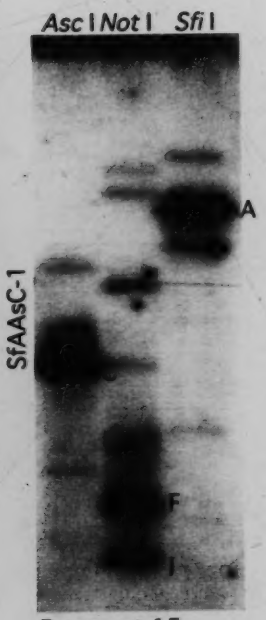
Figure 14. Autoradiographs of cross-hybridized fragments excised from double-digest PFGE gels. The enzyme used to generate each lane is identified at the top of each autoradiograph. The probes corresponding to the fragment numbers in Figures 10,12 and 13 are identified at the bottom of each autoradiograph. The probes are also identified by fragment designation on the left side of each autoradiograph.


Asc I Not I Sfi I
AsC-2
Fragment 7
Asc I Not I Sfi I
AsC-1NoB
Fragment 8
Asc I Not I Sfi I
AsF,NoG
Fragment 9
Asc I Not I Sfi I
AsC-1NoF,AsANoB
Fragment 10
Asc I Not I Sfi I
AsH,AsC-3NoH
Fragment 11
Asc I Not I Sfi I
NoI
Fragment 12



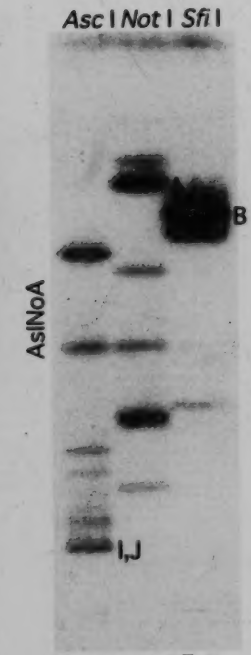
Fragment 13

Fragment 14

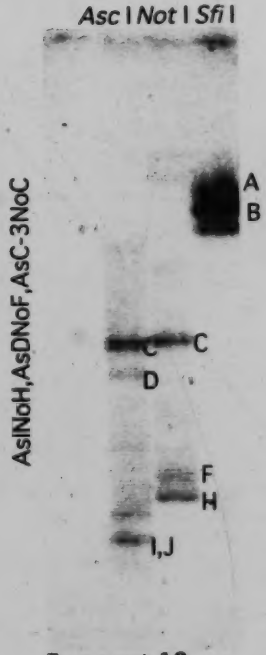


Fragment 15

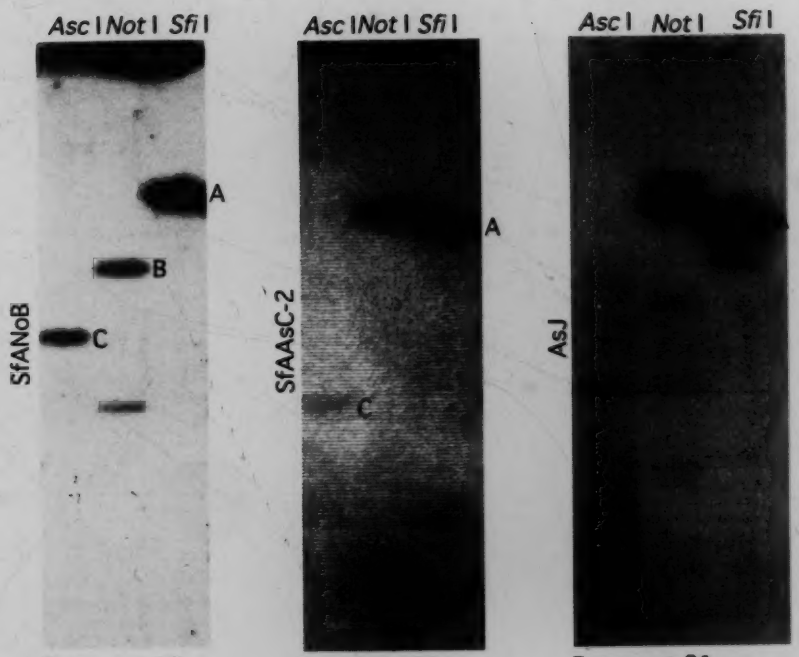
Fragment 16



Fragment 17



Fragment 18



Fragment 19 Fragment 20 Fragment 21

5.5. Hybridization of cloned genes and sequences onto PFGE gels of single and double digests of genomic DNA

5.5.1. locations of rRNA genes

I-*Ceu*I fragments were hybridized on to gels containing *Asc*I, *Not*I, and *Sfi*I fragments (Figure 5 and Table 3). The positions of the ends of the I-*Ceu*I fragments marked the positions of the 23S rRNA genes on the map. The probe for the 16S rRNA gene was hybridized to gels containing single and double-digest fragments of *Asc*I, *Not*I, and *Sfi*I (Figure 15 and Table 5). In Figure 1, the 6 copies of 16S rRNA are identified as 1 through 6 in a counterclockwise direction on the map. When these were compared to positions of 23S rRNA genes on the map, copies 2 through 6 occurred together on the chromosome and copy 1 fell on an area of the map that had not been ordered, AsE and AsH. The 16S rRNA gene hybridized to AsE. CeB hybridized to AsA, H and E. CeA showed no hybridization to AsE or H, likely due to the large size of CeA. Since the order of AsE and AsH is therefore unknown, the 23S rRNA gene (copy 1) could be located on either AsE or AsH.

5.5.2. locations of cloned genes

Figures 16, 17 and 18 and Table 5 show the results of hybridization of the *hhdB,A* (hemolysin gene), *hupA* (hemoglobin binding protein gene), *momp* (OmpA homologue gene) and *ftpA* (fine tangled pilus gene) to gels containing single and double-digest fragments.

Figure 15. Hybridization of 16S rRNA gene probe. The enzymes used to generate each lane on the PFGE gel (A.) and autoradiograph (B.) are identified. The asterisk indicate those bands to which the 16S rRNA probe hybridized and match the labelled bands in B. Table 5 lists the copy number of 16S rRNA which hybridized to each band. P1 and P2 are partial digest bands used to order fragments AsD, B, J and F.

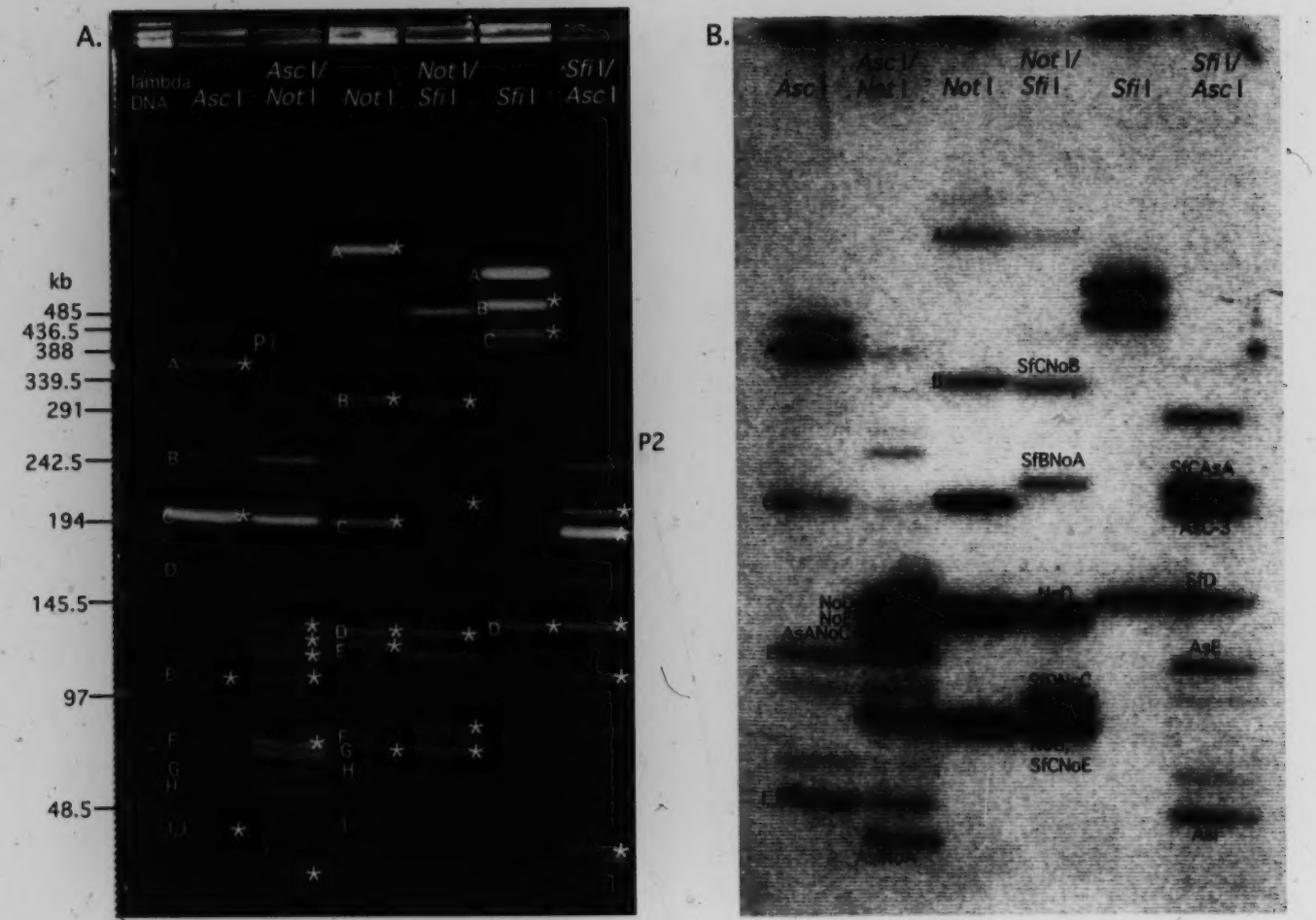

A.
lambda DNA
Asc I
Asc I/ Not I
Not I
Not I/ Sfi I
Sfi I
Sfi I/ Asc I
kb
485
436.5
388
339.5
291
242.5
194
145.5
97
48.5
P1
P2
B.
Asc I
Asc I/ Not I
Not I
Not I/ Sfi I
Sfi I
Sfi I/ Asc I
SfCNoB
SfBNoA
SfCAsA
SfD
AsE
SfCNoE

Figure 16. Hybridization of ***hhdB*** and ***hhdA***, the hemolysin gene probe. The enzymes used to generate each lane on the PFGE gel (A.) and autoradiograph (B.) are identified. The asterisk indicate those bands to which the ***hhdB,A*** probe hybridized and match the labelled bands on (B). P1 and P2 are partial digest bands used to order fragments AsD, B, J and F.

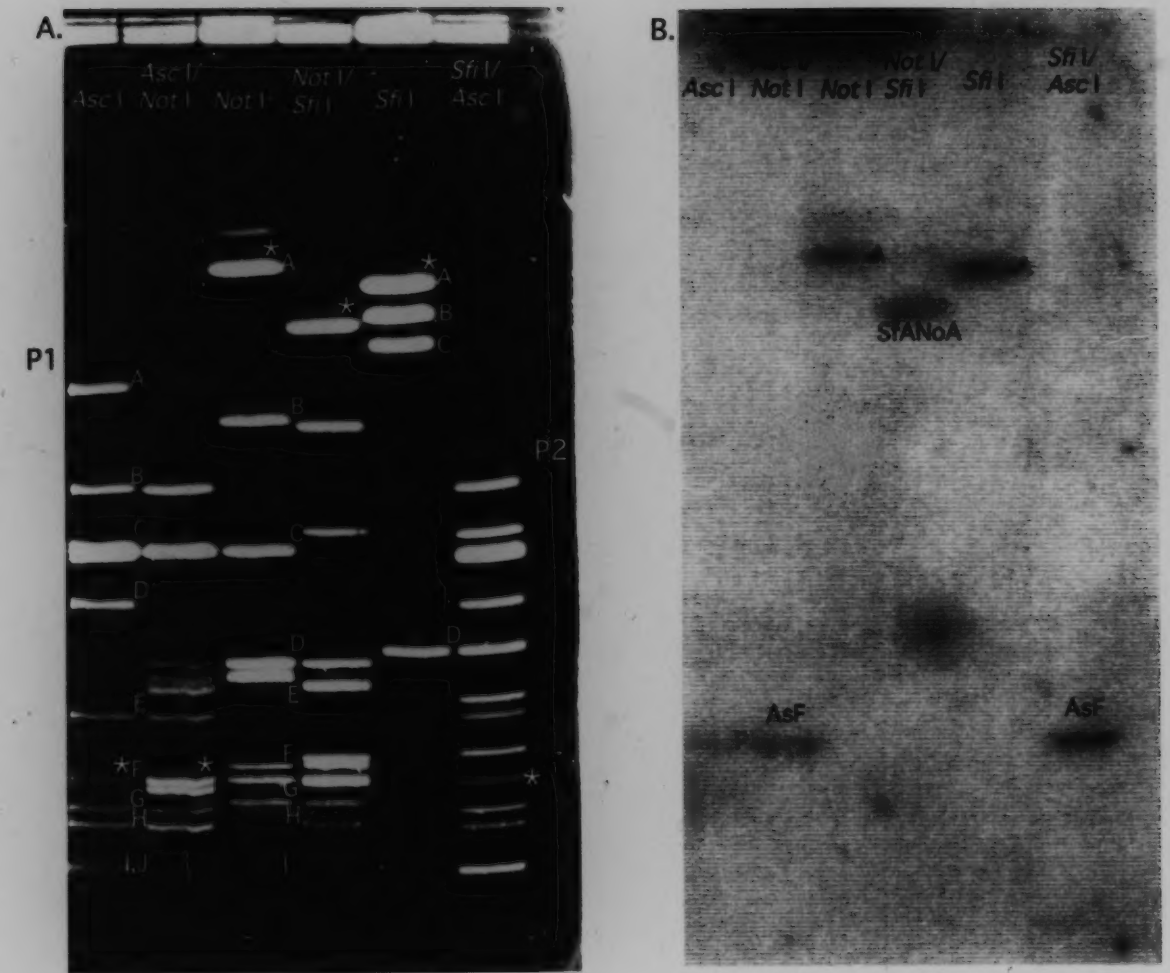

A.
Asc I
Asc I/ Not I
Not I
Not I/ Sfi I
Sfi I
Sfi I/ Asc I
P1
P2
B.
Asc I
Not I
Not I
Not I/ Sfi I
Sfi I
Sfi I/ Asc I
StANoA
AsF
AsF

Figure 17. Hybridization of *hupA* gene encoding hemoglobin binding protein. The enzymes used to generate each lane on the PFGE gel (A.) and autoradiograph (B.) are identified. The asterisk indicate those bands to which the *hupA* probe hybridized and match the labelled bands on (B). P1 and P2 are partial digest bands used to order fragments AsD, B, J and F.

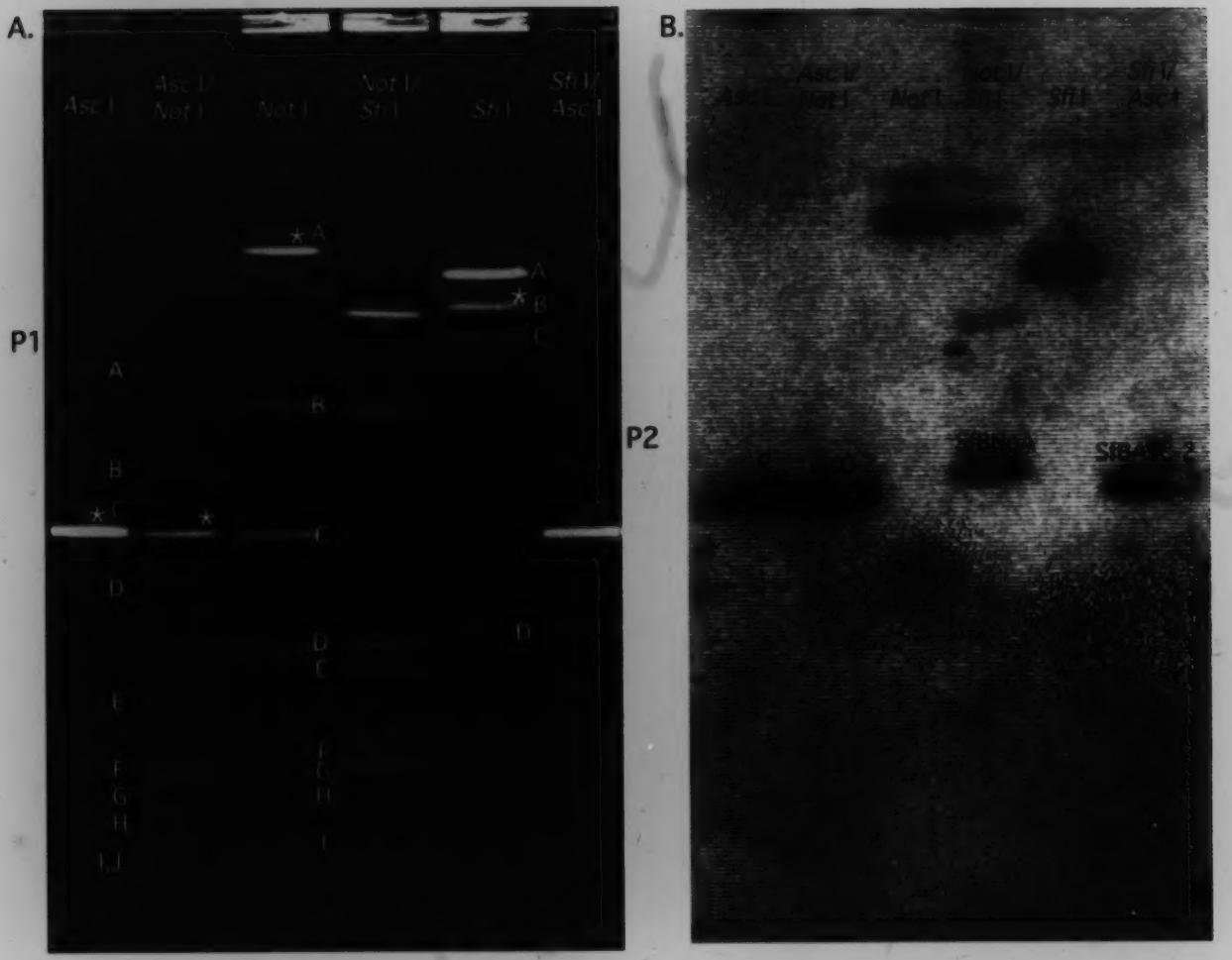
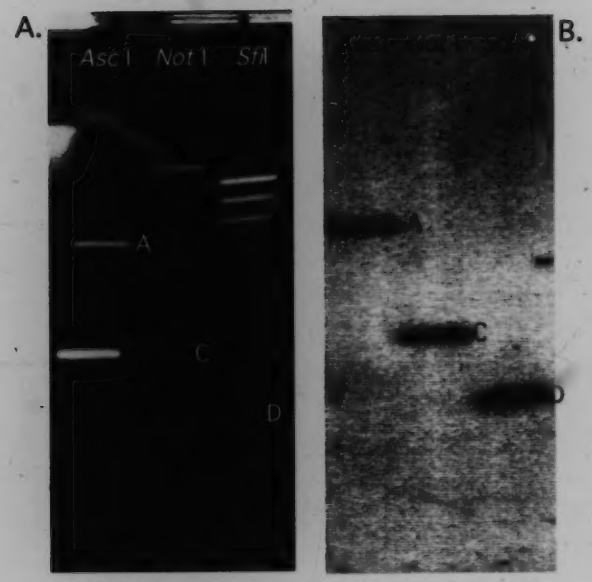
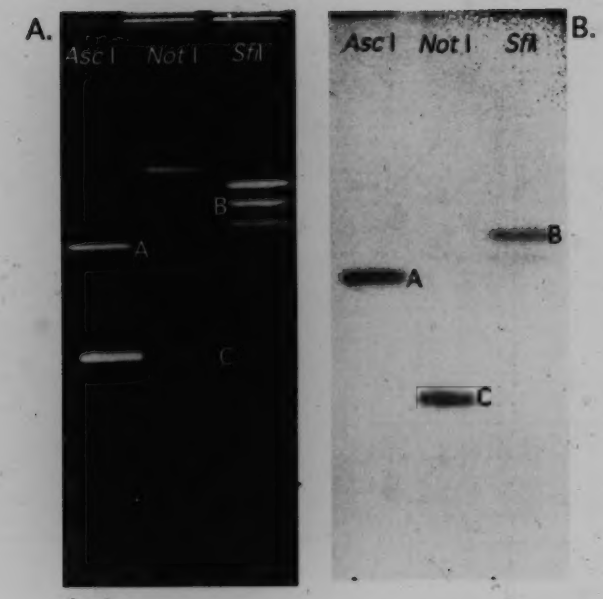

A.
B.
Asc I
Asc I / Not I
Not I
Not I / Sfi I
Sfi I
Sfi I / Asc I
P1
P2

Figure 18. Hybridization of *momp* gene probe encoding OmpA homologue and *ftpA* gene probe encoding fine tangled pili. The enzymes used to generate each lane on the PFGE gel (A.) and autoradiograph (B.) are identified. The bands to which the probe hybridized are labelled.


A.
Asc I
Not I
Sfi I
A
C
D
B.
C
D

momp


A.
Asc I
Not I
Sfi I
B
A
C
B.
Asc I
Not I
Sfi I
B
A
C

ftpA

TABLE 5. Hybridization of cloned genes

PROBE	RESTRICTION ENZYME FRAGMENT PROFILE					
	AscI	*AscI/NotI*	*NotI*	*NotI/SfiI*	*SfiI*	*SfiI/AscI*
16S rRNA[a]						
1	E	AsE	B	SfCNoB	C	AsE
2	A	NoG	G	NoG	C	SfCAsA
3	A	NoE	E	SfCNoE	C	SfCAsA
4	A	AsANoC	C	SfDNoC	D	SfD
5	C	NoD	D	NoD	B	AsC-3
6	I,J	AsINoA	A	SfBNoA	B	AsI
hhdB,A[b]	F	AsF	A	SfANoA	A	AsF
hupA[c]	C	AsC-2	A	SfBNoA	B	SfBAsC-2
momp[d]	A	ND	C	ND	D	ND
ftpA[d]	A	ND	C	ND	B	ND

[a] Figure 15. 16S rRNA copies are labelled 1 through 6 on the physical map (Figure 1, page 30)

[b] Figure 16

[c] Figure 17

[d] Figure 18

5.6. Restriction fragment profiles of CIP 542^T obtained from W. Albritton (KC1) and ATCC.

For this thesis project CIP 542 (KC1) was obtained from W. Albritton. For comparison purposes, CIP 542 was also obtained from ATCC.

Figure 19 illustrates the difference in restriction enzyme fragment profiles of the two strains referred to as *H. ducreyi* CIP 542^T. Band sizes produced by digestion of CIP 542^T chromosomal DNA obtained from ATCC by *Asc*I, *Not*I and *Sfi*I are as follows: *Asc*I; 352, 210, 180, 145 (doublet), 128, 95, 78, 36 and 31 kb. *Not*I; 430, 291, 240, 140, 133 (doublet), 105 (doublet) and 65 kb, *Sfi*I; 630, 582, 302 and 133 kb.

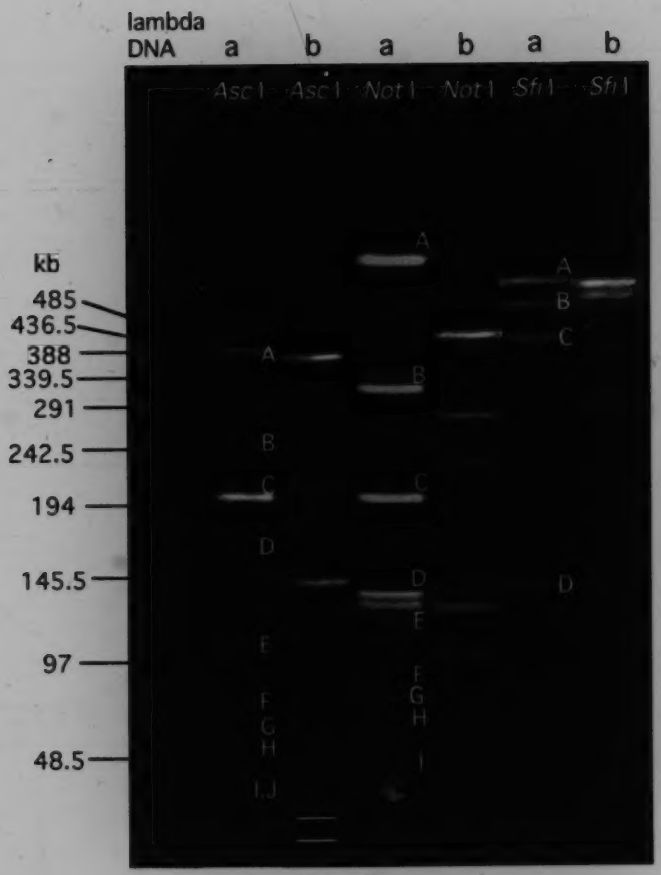


Figure 19. Comparison of enzyme profiles of two strains referred to as *H. ducreyi* CIP 542^T. PFGE separation of *Asc*I, *Not*I and *Sfi*I digestion fragments of *H. ducreyi* CIP 542^T chromosomal DNA from cultures supplied from: lane a; W. Albritton (KC1) and lane b; ATTC (#33940). The two lines at the bottom of *Asc*I lane b indicate positions of two faint bands. Band sizes of the lambda DNA concatemers used as size standards are indicated.

6. DISCUSSION

6.1. Overview

The physical genome map was constructed using pulsed-field gel electrophoresis, a powerful technique that has been applied to many organisms. This "top-down" approach has been extensively reviewed (Cole *et al.*, 1994, Fonstein *et al.*,1995, Smith *et al.*, 1990). PFGE for physical mapping applied particularly well to *H. ducreyi* since genetic transfer systems are not well established for this organism. Four restriction enzymes, *Sfi*I, *Not*I, *Asc*I and I-*Ceu*I, were used to produce mapping intervals in the range of 10 to 250 kb with a total of 31 restriction sites on the chromosome. The largest interval of 250 kb occurred only once on the genome. This physical map is therefore adequate for positioning genes as well as for epidemiological and taxonomic purposes (Cole *et al.*, 1994). The size of the genome was determined to be 1771 ± 43 kb with circular geometry.

Construction of the physical map first required restriction enzymes that cut the genome infrequently and produced easily resolveable fragments by PFGE. In searching for restriction enzymes, the fact that *H. ducreyi* has a guanine-plus-cytosine content of 38 to 39 mol% was used (Casin *et al.*, 1985). *Asc*I, *Not*I and *Sfi*I are restriction enzymes with 8-bp specificity having GC-rich recognition sites and were found to generate a consistent and appropriately limited number of restriction fragments. I-*Ceu*I has a 26 bp restriction site that is conserved in the 23S rRNA of many eubacteria (Liu *et al.*, 1993). *Apa*I, *Sma*I and *Rsr*II were used to create the map of *H. influenzae* Rd (Lee *et al.*, 1989). These enzymes produced either inconsistent results or too many fragments with *H. ducreyi*.

Cross-hybridization and double digestion are common mapping strategies (Cole *et al.*, 1994) and were used to create maps of *H. influenzae* and *parainfluenzae* among others. The major difficulty with this approach is non-specific hybridization presumably caused by repeated sequences (e.g. rRNA operons) and contamination of isolated restriction fragments with small amounts of total genomic DNA. Additional information from double and sequential digests and from cross-hybridizing of fragments from these digests were used to create the map. Double digests proved to be an excellent method in that all fragments could be identified when compared to single digests. This enabled the precise positioning of restriction sites when overlapping fragments existed. Mapping of the clones did not link any of the fragments but confirmed the organization of the map.

6.2. Comparison of physical and genetic maps: *H ducreyi* strains CIP 542^T and 35000

Recently, a rudimentary restriction map of the virulent 35000 strain was published using the two enzymes I-*Ceu*I and *Sfi*I (Hobbs *et al.*, 1996). *Not*I did not digest strain 35000 DNA but we found consistently good results with *Not*I using strain CIP 542 (KC1). *Asc*I produced 12 bands with several unresolved triplets or doublets for strain 35000 and was not used (Hobbs *et al.*, 1996). *Asc*I produced 10 bands with one unresolved triplet with CIP 542. The triplet poses no problem in the mapping of genes since AsC-1, 2 and 3 do not overlap any common *Not*I fragments. *Asc*I was important in improving resolution of the CIP 542 map since it produced restriction sites within the double digest fragment SfANoA, a large section of 495 kb. These two maps now allow the direct comparison of a virulent and avirulent strain

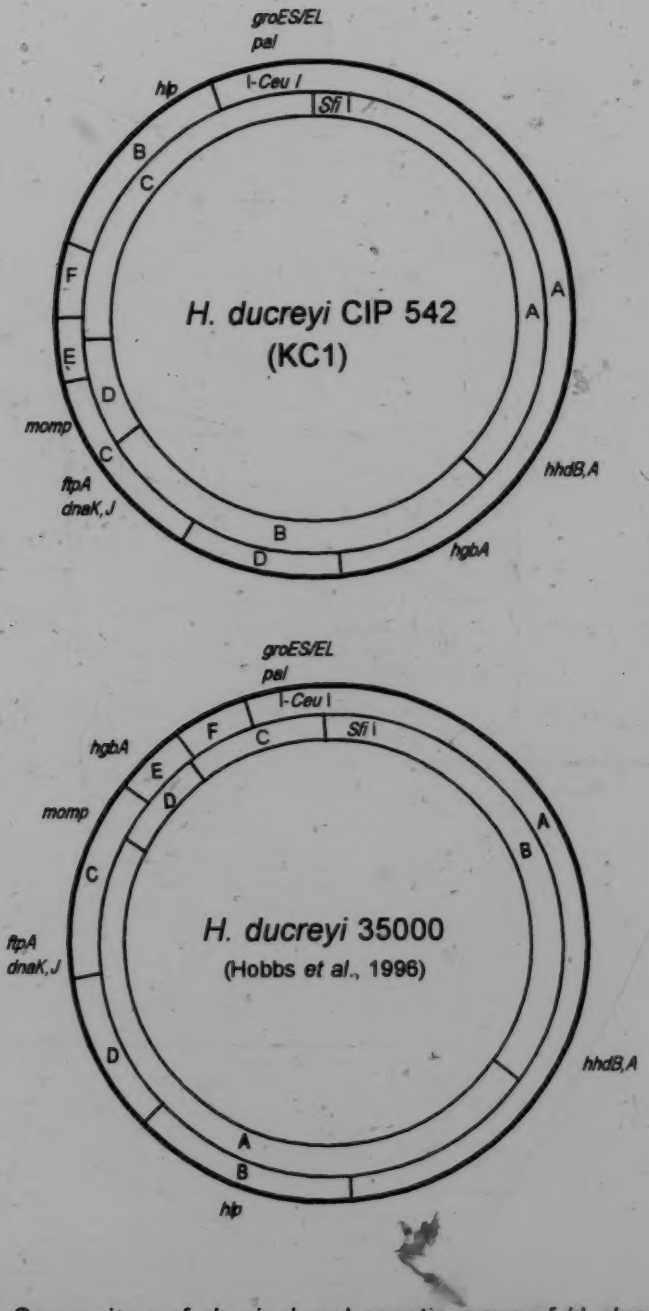


Figure 20. Comparison of physical and genetic maps of *H. ducreyi* CIP 542 (KC1) and 35000 (ATCC 33922). Both strains have I-*Ceu*I fragments of the same size but have different *Sfi* I fragment sizes.

by genome size, restriction fragment profile, fragment order on a physical genome map and gene order (Figure 20).

Changes in restriction fragment profile with I-*Ceu*I digestion allow the easy detection of chromosomal rearrangements (due to insertions or inversions) and are useful in studying genetic diversity within species or among closely related species. This is due to the conservation of rRNA genes both in number and in position on the chromosome and the specificity with which I-*Ceu*I cuts the *rrn* site. (Liu *et al.*, 1995). Therefore, it is not surprising that both strains 35000 and CIP 542^T (ATCC 33940) displayed identical I-*Ceu*I fingerprints (Hobbs *et al.*, 1996). CIP 542^T (KC1) also displayed identical I-*Ceu*I fingerprints to that published by Hobbs et al of strain 35000. This suggests that there is conservation of the genome between the two strains: both have 6 *rrn* genes and the same size of chromosome fragment between the *rrn* genes. It is surprising, however, that the order of the I-*Ceu*I fragments differed between the two strains. The order of I-*Ceu*I fragments clockwise around the chromosome for 35000 is A-B-D-C-E-F and A-D-C-E-F-B for CIP 542^T, i.e. a rearrangement has occurred in the movement of CeB. In both strains *pal* and *groE* are positioned at the left end of CeA and *hhdB,A* at the opposite end of the fragment (using the resolution of the CIP 542^T map *hhdB,A* hybridizes to AsF and therefore maps at the end of CeC but this cannot be assumed for 35000). In both strains *ftpA* and *dnaK,J* are located together on CeC followed by *momp*. The *hlp* gene is located on CeB in both strains. Since the postion of CeB is different for the two strains, the position of *hlp* is also inverted. The only gene that hybridizes to different fragments in the two strains is the hemoglobin receptor gene (referred to as *hgbA* by Hobbs *et al* and *hupA* in our study reflecting probe sources) which is located on CeE in 35000 and CeA in CIP 542. Interpretation of this is

affected by some limitations as discussed by others (Casjens *et al.*, 1995, Liu *et al.*, 1995). For example, the *rrn* genes are not necessarily homologous. Assuming they are, and the origin of replication (*oriC*) is located on CeE as suggested for strain 35000 (Hobbs et al.,1996) this means that the hemoglobin receptor gene has moved away from *oriC* in strain CIP 542^T. Essential genes are clustered around *oriC* to ensure efficient transcription (Schmid *et al.*, 1987, Ladefoged *et al.*, 1992). The hemoglobin receptor is essential to the ability of the organism to use hemoglobin and hemoglobin-haptoglobin as a source of heme and is required for virulence in an animal model (Stevens *et al.*, 1996). Movement of genes away from *oriC* affects average gene dosage (Schmid *et al.*, 1987) which is perhaps related to the reduced virulence of strain CIP 542^T. Another possibility is that *oriC* is located on CeA in strain CIP 542^T and that the *rrn* genes are not homologous.

Differences in the two maps point to genetic diversity which has not yet been explored in *H.ducreyi* partly due to the lack of a suitable typing system (Trees *et al.*, 1995). It is also possible that rearrangements may be the result of lab adaptation similar to the situation that has been found to exist in enteric bacterial cultures (Hill *et al.*, 1988). We have observed changes in *Sfi*I band sizes with serial passage of cultures (unpublished). This observation is again reflected in the change in order of *Sfi*I bands between the two maps. Of note as well, the I-*Ceu*I fragments did not hybridize to the same *Sfi*I fragments when comparing the two maps, but ultimately it is comparison of gene order between the strains that will identify chromosomal rearrangements and genetic diversity. To summarize the gene order differences between the two maps, the order of outer membrane lipoprotein gene (*hlp*) and hemoglobin receptor gene (*hgbA* or *hupA)* is

inverted while the order of *ftpA*, *dnaK,J* and *momp* genes remained the same but moved closer to the *groE* and *pal* genes on strain 35000.

6.3. Genome size

The size of the CIP 542 genome is small, about 38% the size of the *E.coli* genome (Kohara *et al.*, 1987). The size of the *H. ducreyi* genome likely reflects its fastidious nature and limited biochemical activity (Morse, 1989). *Campylobacter* species (Chang *et al.*, 1990), *Helicobacter pylori* (Taylor *et al.*, 1992), *Haemophilus influenzae* RD (Lee *et al.*, 1989) and *Rickettsiella melolanthae* (Frutos *et al.*, 1989) have genome sizes similar to that of *H. ducreyi*.

6.4. *rrn* loci

H.ducreyi has 6 copies of 16S rRNA, the same number as *H. influenzae* (Lee *et al.*, 1989). *rrn* copy number ranges from 1 in *Mycobacterium leprae* (Sela *et al.*, 1989), 1 in *Mycoplasma gallisepticum* (Chen *et al.*, 1989) 2 in *Treponema denticola* (MacDougall, *et al.*, 1995) to 10 in *Bacillus subtilis* (Loughney *et al.*, 1982) , with *E. coli* (Bachmann, 1990) and *Salmonella typhimurium* (Liu *et al.*, 1993) having 7 copies. The number of rRNA operons relates to generation time and a comparison can be made with *H. influenzae* which has a generation time of 29 min in rich medium (Kauc *et al.*, 1989) compared with 1.8 to 4 h for *H.ducreyi* (Neidhardt *et al.*, 1990). The six rRNA loci are located in a region of 888 kb, which accounts for 50% of the chromosome. This is similar to the situation in bacteria such as *H. influenzae* (Lee *et al.*, 1989), *E. coli* (Bachmann, 1990), *Bacillus subtilis* (Piggot, 1990) and *Clostridium perfringens* (Canard *et al.*, 1989) which have *rrn* loci grouped within an area occupying 30-50% of the genome.

The 16S rRNA gene was mapped using a homologous gene probe. The ends of the I-*Ceu*I fragments marked the positions of the 23S rRNA genes. The 16S and 23S rRNA loci were located on the same fragments of the map for 5 of the 6 copies. Copy 1 (Table 5, page 77) of the 16S rRNA gene was located on AsE, an area of the map which has not been ordered so that its position on the same fragment as the 23S rRNA gene could not be confirmed. 16S, 23S and 5S rRNA genes are often located within the same operon (Krawiec, S *et al.*, 1990) but this is not always the case. For example, strains of *Campylobacter jejuni* (Newnham *et al.*, 1996) and *Helicobacter pylori* (Jiang *et al.*, 1996) have the 16S and 23S rRNA genes located separately on the chromosome.

6.5. Mapping of genes

Twenty-six genes or DNA sequences were mapped and are scattered around the CIP 542 chromosome. The hemolysin gene (Totten *et al.*, 1995, Palmer *et al.*, 1996) was obtained from two sources and, as expected, mapped to the same position. Likewise, partial sequences of the major outer membrane protein gene, *momp*, OMP A homologue (Hiltke *et al.*, 1995) were obtained from two sources (E. Hansen and S. Spinola) and both hybridized to the same area of the map. The UDP-glucose epimerase gene encoding an enzyme involved in the biosynthesis of LOS hybridized to two areas of the map. This indicates that two copies of this gene may be present in *H. ducreyi*.

6.6. Comparison of restriction enzyme fragment profiles of CIP 542 (KC1) and CIP 542 (ATCC #33940)

The restriction fragment profiles of CIP 542^T supplied by W. Albritton (KC1) and ATTC 33940 were compared using the criteria proposed by Tenover *et al* (Tenover *et al.*, 1995), and found to be different strains. Fewer than 50% of fragments are common in all 3 restriction enzyme fragment profiles. This could be an important point since confusion has arisen in the definition of virulence, since the avirulent CIP 542 has proven to be virulent in some models (Trees *et al.*, 1991, Trees *et al.*, 1992). At least 2 different strains are currently referred to as the type strain, CIP 542, and this disparity may help clarify some confusion regarding virulence of this strain.

7. CONCLUSION AND FUTURE STUDIES

In conclusion, the goals of this thesis project have been accomplished. A physical genome map of the type strain of the species was constructed using 4 restriction enzymes which marked 31 restriction sites on the chromosome and produced mapping intervals of 10 to 250 kb. The genome size was determined to be 1771 ± 43 kb. The 16S rRNA genes and 26 other homologous genes or sequences were mapped. Fragments AsH and E remain unordered on the map. Ordering could be done by performing a double digest of *Asc*I and I-*Ceu*I to determine if AsH or E harbours the I-*Ceu*I restriction site and therefore the 23S rRNA gene. The logical extension to this work would be to analyze the *Asc*I/I-*Ceu*I double digest to determine the exact sizes of the overlapping fragment, as has been done for the other combinations of double digests using *Asc*I, *Not*I and *Sfi*I.

Future studies should include regular updated versions of the map to include newly published genes of *H. ducreyi*. Additionally, this thesis has shown that at least two strains of *H. ducreyi* have been designated CIP 542, the KC1 strain and the ATCC #33940 strain. Those working with the strain should identify it using restriction fragment profiles to clarify which strain they are using.

Now that a map of *H. ducreyi* CIP 542^T has been constructed, its uses as outlined in the introduction will become evident. In the field of pathogenesis, a comparison of a virulent strain 35000 (Hobbs *et al*, 1996) and the avirulent strain CIP 542^T has already begun with this thesis. To improve on this comparison which included only the two enzymes I-*Ceu*I and *Sfi*I, more work needs to be done on the strain 35000 physical genome map to include

the enzymes *Not*I and *Asc*I. *Not*I did not digest the chromosomal DNA of strain 35000 (Hobbs et al, 1996) and this needs to be confirmed using the methods described in this thesis. If chromosomal DNA of strain 35000 is not digested by *Not*I, this would indicate a fundamental difference between the two strains. Chromosomal rearrangement is already evident from differences in fragment and gene order between the two strains. Ultimately, these studies will contribute to our understanding of the physiology and regulation of pathogenesis in *H. ducreyi*.

As was summarized in section 2.5, a variety of typing schemes have been proposed for *H. ducreyi* however their usefullness has not been determined. Comparing strains according to type and genome map would be a rigorous method of proving the level at which the strains are being distinguished from each other. Related to this is the study of the epidemiology of the disease.

The task of reorganizing the genus *Haemophilus* could be simplified using the genome maps of a variety of strains of *H. ducreyi*. The phylogenetic relationships of members of the genus *Haemophilus* could be studied using genome maps since they contribute greatly to information on genomic organization and rearrangements.

Genetic diversity is an unexplored topic for *H. ducreyi*. As discussed above, the differences in maps between strain 35000 and CIP 542 are an indication of potential genetic diversity within the species. By comparing gene order and the locations of genes with respect to restriction sites, *H. ducreyi* strains may prove to be as genetically similar as the *E. coli*, *Shigella*

flexnerii, and *Salmonella typhimurium* group (Krawiec, *et al.*, 1990) or as genetically diverse as *Helicobacter pylori* (Jiang *et al.*, 1996).

Since a map of CIP 542^T is now available, this strain would be a useful candidate for whole genome sequencing, as has been published for *H. influenzae* Rd (Fleischmann *et al.*, 1995). However, the study of how an organism causes disease necessitates the comparison of virulent and avirulent strains and it would be impossible to sequence large numbers of strains to determine how they differ. Additionally, DNA sequences do not necessarily define function (Strauss *et al.*, 1997). Comparing physical genome maps, gene order and positions of genes with respect to restriction sites remains a valuable tool that can be used to augment whole genome sequencing.

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